
3rd Conference on Experimental and Translational Oncology
Tretja konferenca o eksperimentalni in translacijski onkologiji

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**3rd Conference on Experimental and Translational Oncology**
Kranjska gora, Slovenia,
March, 18-21, 2004

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L19. Christoph Peters: Role of lysosomal cysteine peptidases in tumour progression

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L21. Rolf Bjerkvig: Angiogenesis independent brain tumour growth

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# ABSTRACTS OF LECTURES

# Proteolysis and cancer - not just tumor cells anymore

*Bonnie F. Sloane, Mansoureh Sameni, Julie Dosescu, Stefanie Roshy, Izabela Podgorski and Kamiar Moin*

Department of Pharmacology and Barbara Ann Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201 USA

The clinical trials on inhibitors of the matrix metalloproteases (MMPs) did not fulfill the promise of MMPs as therapeutic targets in cancer. This was a surprise as the preclinical data implicating MMPs in malignant progression was compelling. There are several possible explanations for the 'disconnect' between the preclinical and clinical data. Thoughtful and thought-provoking reviews on this topic have been published by Coussens et al. (Science 295:2387, 2002) and Egeblad and Werb (Nature Reviews Cancer 2:161, 2002). Among the critical issues that have arisen from the problems encountered in the MMP inhibitor (MMPI) trials is whether the MMPIs actually inhibited their target MMPs *in vivo*, whether the MMPIs targeted MMPs that were causally involved in malignant progression and in this regard whether MMPs might play negative as well as positive roles in malignant progression. Other issues are whether the MMPs targeted by the MMPIs were actually expressed in the tumors studied in the clinical trials, whether proteases other than MMPs should be targeted, and when MMPIs or inhibitors of other proteases might be most effective against tumors. When to use protease inhibitors as therapeutics is affected in part by the relative roles of tumor cells and tumor-associated cells at various stages of malignant progression, yet oncologists have tended to think of therapies as being directed solely against tumor cells. This has in fact been the basis of therapies using cytotoxic drugs, radiation or surgery.

Directing therapies against tumor cells may well have contributed to the clinical failure of MMPIs as it has long been known that many proteases are produced by normal cells associated with the tumors, including fibroblasts, inflammatory cells, and endothelial cells, rather than tumor cells. This is true for MMPs, many of which are produced by fibroblasts, and also for serine proteases such as urokinase plasminogen activator (uPA), which in some cancers is produced by fibroblasts and binds to its receptor on the surface of the tumor cells. Other serine proteases come from tumor-associated inflammatory cells (e.g., macrophages, mast cells, neutrophils). Tumor cells and tumor-associated cells, on the other hand, often express cysteine proteases. For example, cathepsin B is highly expressed in both tumor cells and infiltrating macrophages in colon and prostate tumors. Added evidence as to the importance of non-tumor cells in regard to proteases and malignant progression comes from recent preclinical studies on the contribution of cysteine proteases to malignant progression in transgenic models, studies that have established causal roles for macrophage cysteine proteases or specifically macrophage cathepsin B.

In an effort to evaluate the roles of proteases from various cells in tumor proteolysis, we are developing methodologies and probes for imaging protease activity *in situ* in live tumor cells alone and in co-cultures with tumor-associated cells and *in vivo* in transgenic mouse tumor models and in human tumor xenografts in immunodeficient mice. Such methodologies and probes should be useful for screening the efficacy of protease inhibitors against their target proteases *in vitro* and in vivo as well as for assessing the ability of the protease inhibitors to reach and inhibit their target proteases *in vivo*. In short, such assays could serve as surrogate endpoints in future clinical trials with protease inhibitors as could similar approaches in clinical trials of other non-cytotoxic therapeutic agents.

# Proteases and their inhibition in the development of metastases

*Cornelis J.F. Van Noorden*

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Proteases are considered to be attractive targets for therapeutic strategies in cancer. Especially, invasion and metastasis of cancer are processes in which proteases play a role. Proteases are a class of enzymes that are suited for the design of selective small-molecular irreversible inhibitors. For successful application of protease inhibitors, it is essential to know which proteases are involved in what steps in invasion and metastasis and whether their involvement is essential. Classes of proteases that have been related with cancer progression are cathepsines, plasminogen activators and matrix metalloproteinases. All these proteases are synthetized in a (pre)proform and need posttranslational activation before they are functional. Moreover, the balance between endogenous inhibitors and proteases determines whether they are active. Therefore, mRNA or protein profiles are not informative enough to determine whether a protease is involved in cancer progression and it is essential that protease activity is determined. Furthermore, a link between protease activity and invasion and/or degradation of extracellular matrix pinpoints the protease to be involved. Therefore, we analyze microscopically in animal models which activity of proteases is structurally linked with invasion and metastasis of cancer. We have found activity of cathepsin B, urokinase-type plasminogen activator and gelatinases (MMP-2 and MMP-9) to be involved in tumor development of colon cancer cells in rat liver and development of human pancreatic adenocarcinomas in nude mice. However, treatment of the animals with selective inhibitors of these proteases had partial effects only, indicating that the role of a particular protease can be taken over by other proteases. *In vivo* use of less selective protease inhibitors does not seem to be an option because of side effects as proteases are involved in many physiological processes but combinations of two or more selective inhibitors against different proteases may be the way to go for the development of therapies. Alternatively, inhibition of proteases that are not expressed by cancer cells but are involved in tumor development may be attractive, because cancer cells are genetically instable and therefore, expression profiles including those of proteases can change rapidly. The most promising approach at the moment is inhibition of proteases involved in invasion of endothelial cells that occurs during angiogenesis. It is concluded that the initial therapeutic strategy to block single proteases involved in cancer progression is not sufficient but proteases remain important targets for specific therapy in cancer.

# Lysosomal pathways to apoptosis: role of papain-like cysteine proteases

*Boris Turk*

Dept. Biochem. & Mol. Biol., Jožef Stefan Institute, Jamova 39 Ljubljana, Slovenia

Apoptosis is the major way of eliminating potentially harmfull and excessive cells. The pathway is severely impaired in cancer and cancer cells generally fail to die. A number of events in apoptosis is governed by proteolysis with caspases playing the major role. Recently, evidence has been provided that lysosomal proteases, the cathepsins, are linked with apoptosis. The most studied cathepsins in this respect are cathepsin B, which is a cysteine protease, and the aspartic protease cathepsin D. We were able to show that cathepsins can activate caspases indirectly via proteolytic cleavage of the proapoptotic Bcl-2 homologue Bid at a site distinct fom those of caspase-8 and granzyme B. The entrance point into the apoptotic cascade thus lies upstream of mitchondria. However, in cancer increased cathepsin levels often are connected with poor prognosis, leading to an apparent discrepancy, which will be discussed.

# Multiple functions of lysosomal proteolytic enzymes in cancer

*Simon Caserman, Miha Trinkaus, Nataša Sever, Irena Zajc, and Tamara Lah Turnšek*

Department of Genetic Toxicology and Cancer Biology, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia

Lysosomal proteinases - cathepsins (Cats) are composed of different members from the peptidase families: the aspartic family (A4-A26, Cats D, E) the cysteine family (C1-C63, Cats B, H, L, S, C, K, O, F, V, X and W) and the serine family (S8-S50, Cats A, G). In the last two decades, it has become clear that lysosomal proteases, such as Cat D and Cats B, H, L, S and possibly K and O are involved in the progression of various tumours, being over expressed and some of them translocated to plasma membrane or extracellular environment in abnormally high amounts in many different neoplastic tissues. They are unique not only because of their universal localisation, but also due to their selective specificity of numerous protein substrates. They are active in the pH range from 4 - 8, depending on their microenvironment. Cysteine Cats are ultimately regulated by cysteine protease inhibitors (CPIs) of the cystatin family forming tight, equimolar non-covalent complexes, either in (stefins A, B) or outside the cells (cystatins C, M).

We are investigating three major physiological processes, which play crucial role in cancer progression; these are invasion of tumour cells, tumour cell apoptosis and angiogenesis in tumours. Evidence, that in all three processes cysteine cathepsins play certain roles will be presented in the studies in intracranial tumours (meningioma, glioma, human hypophysis and pituitary adenomas) and breast carcinoma.

Our results *in vivo* and *in vitro* indicate that elevated levels of CatB in breast and intracranial tumours are associated with increased tumour invasiveness. In apoptosis, the roles of CatB and CatL seem to be opposite, as pro- and anti-apoptotic activities were observed in various systems, including our data in glioblastoma cell lines, where CatL enhanced their resistance to staurosporine induced cell death. Angiogenesis *in vivo* seems to be associated with high expression of CatB but not CatL. In *in vitro* angiogenesis assay (the rat aorta ring assay), carried out in the presence of general cysteine cathepsins inhibitor E64, capillary sprouting was not significantly effected, but collagen degradation during the regression phase of the experiment, was partially inhibited. Cat L was up regulated, but Cat B was downregulated in human dermal micro-vascular endothelial cells during their induced growth on collagen gel, indicating possible yet unknown roles of the cathepsins in cell proliferation. Evidence of altered levels of endogenous inhibitors, stefins A and B, in advanced tumour stages will also be presented. The presented data support the development of anti proteolytic therapy, including inhibitors of these two cysteine cathepsins.

# Degradome profiling in breast and prostate cancer

*Dylan R. Edwards*

School of Biological sciences, University of East Anglia, Norwich, NR4 7TJ.

The involvement of proteases in cancer biology has long been attributed to their ability to promote tumour cell invasion and metastasis. However, it is now clear that proteases affect many distinct processes that impact on tumour growth, including the regulation of cell proliferation, adhesion and apoptosis, as well as angiogenesis and immune surveillance. Proteases do not always promote tumouriogenesis, as there are situations where proteolytic activity serves to hold tumours in check. But the notion of proteases as targets for cancer therapy is still viable, though it is necessary to define more precisely the key enzymes of the "Degradome" - the repertoire of proteases that a cell or tissue deploys - involved in execution of specific functions during tumour progression. We have therefore set about analysis of the expression of ~100 protease and related genes using TaqMan real-time RT-PCR in breast and prostate tumour tissues. To date our work has focussed on expression profiling of the matrix metalloproteinases (MMPs), adamalysins (ADAMs), adamalysin-thrombospondins (ADAMTS) families, as well as their inhibitors and components of serine protease networks. Analysis of surgical specimens has been complemented by use of laser capture microdissection to characterize epithelial versus stromal contributions to expression. In addition, we have used isolated populations of stromal and epithelial cells from normal and tumour tissue to further refine these profiles. In the case of breast cancer, we have also been able to contrast this information with the analysis of primary mammary tumours from PyMT transgenic mice. We will review the current knowledge of protease gene expression in breast and prostate cancer, with particular attention to the identification of novel potential diagnostic or prognostic markers, and key candidate genes for further functional evaluation.

Supported by grant LSHC-CT-2003-503297 from the European Union Framework Programme 6.

# Tumor-associated proteolytic systems: the serine protease family of human tissue kallikreins

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Invasiveness of a variety of solid malignant tumors depends on the regulated expression of proteolytic enzymes that degrade the surrounding extracellular matrix and dissociate cell-cell and/or cell-matrix attachments. In concert with other proteolytic factors such as matrix metalloproteinases or cysteine proteases, the serine protease urokinase/plasmin system acts as one of the key players in these processes. In very recent years, however, another serine protease family, the human tissue kallikreins, has attracted attention, since it has been shown that most of the tissue kallikrein genes/proteins are either under- or overexpressed in certain carcinomas, especially in breast, prostate, testicula, and ovarian cancer. Thus, the tissue kallikreins represent interesting tumor biomarkers and are considered novel therapeutic targets in cancer.

The human tissue kallikrein (KLK) gene family encompasses 15 members, all clustered in a 300 kb region on chromosome 19q13.4. All genes consist of five coding exons, some genes contain in addition one or two 5'-untranslated exons. The intron phases between coding exons are completely conserved among all members, which underlines the similar genomic organization of the KLK genes. Expression of several of the KLK genes appears to be under the control of steroid hormones. The encoded tissue kallikreins (hK1 to hK15) belong to the serine protease family, most of them displaying trypsin-like activity with exception of hK3, hK7, and hK9 which are assigned to the chymotrypsin-like proteases. Tissue kallikreins are expressed as pre/pro-peptides, consisting of a signal peptide driving secretion followed by a rather short activation peptide (with exception of hK5), which has to be cleaved off to generate the enzymatically active mature protein of about 24-28 kDa. In most cases, activation occurs by cleavage after an arginine or lysine residue (with exception of hK4 and hK8 which are activated by cleavage after glutamine). The tissue kallikreins display five (hK1, hK2, hK3, hK13) or six (all the others) highly conserved disulfide bonds.

Most information on the (patho-)physiological role and function is available for the "classical" tissue kallikreins hK1 to hK3. hK1 is the so far only identified tissue kallikrein that acts similar to plasma kallikrein, *i.e.* it does release lysyl-bradykinin (kallidin) from LMW-kininogen. hK3 (well known as prostate specific antigen; PSA) plays an important role in liquefaction of the seminal plasma clot after ejaculation under physiological conditions. In prostate cancer, hK3 is widely used to aid in cancer diagnosis and monitoring of therapy response. hK2 is co-expressed with hK3 and has the potential to activate pro-hK3. The (patho-)physiological functions of the other so-called "new" tissue kallikreins are not yet resolved in detail. The *de novo* expression or up-regulation of at least some of these proteases in different types of cancer, especially ovarian, breast or prostate cancer, indicates that these proteases are part of tumor cell-associated proteolytic cascades aimed at increasing the invasive potential of the tumor cells.

# Gene therapy for liver tumours

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Liver tumours are common in the world. Primary liver tumours, or hepatocellular carcinoma, are prevalent in areas where there is endemic hepatitis B and C. Secondary liver tumours are mainly from colorectal cancer and constitute the large majority of tumours in the Western world. Our group has studied the gene therapy approach for controlling tumour in the liver, as well as trying to stimulate the level of platelets in the blood. This is relevant as most patients with primary hepatocellular carcinoma suffer from liver failure with decrease in platelet count partly due to decrease in thrombopoeitin (TPO) production from the failing liver. Patients with colorectal liver metastases can suffer from thrombocytopaenia following intensive chemotherapy.

In an attempt to control liver tumours we have studied the effect of intravenous, intratumoural and intra-arterial injection of Adeno-E1B deleted virus. This is a conditional replication virus previously described as the "smart bomb", which is designed to replicate in cells deficient of p53. Ascending doses were injected up to $3x10^{10}$ plaque forming units. There were very few treatment related toxicities, mainly in the form of fever. CT scan showed no patient with diminution in the size of the tumour. Serological and immunological studies showed increase in the antibody titre in the serum of patients following injection of adenovirus. Subsequent studies have shown that these antibodies were against penton base. Further studies pinpointed the epitopes responsible for this immune reaction. Future engineering of adenovirus might lead to development of a more suitable adenovirus.

TPO gene therapy was studied in order to stimulate platelet production, which would be useful to treat liver failure in patients with hepatocellular carcinoma or prevent thrombocytopaenia following intensive chemotherapy. First, we investigated the possibility of intramuscular injection of TPO used alone and in combination with electroporation, ultrasound and Optison™.We found that the combination of electroporation and ultrasound with Optison™ led to the maximum TPO production following intramuscular injection. We also observed that the use of plasmid with EF1$\alpha$ leads to longer expression than plasmid with CMV promoter. However, all plasmids failed to increase the serum platelet production. Facing this lack of success with the intramuscular approach we then investigated delivery of the plasmids via the intrahepatic route and by slow intravenous injection. Both approaches were successful in greatly increasing the serum TPO in the blood, megakarocytes in the bone marrow, and platelet count in the systemic circulation. This preliminary study is encouraging, but further refinements are needed before it can be taken to the clinic.

# Up- and down-regulated gene expression in the oncogenesis of gastric cancer: a systemic approach

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The dominant paradigm of the last 25 years has been that tumors grow on account of mutation and expansion that endow the cell with the power to outbreed its neighbors, and it is believed that four to ten mutations in the right genes can transform any cell. According to this theory any malignant transformation occurs through interactions between a series of altered oncogenes and anti-oncogenes. In the literature the list of cancer-related mutations has grown to more than 100 oncogenes and 15 tumor suppressor genes. However, after 7-year study of the molecular biology of gastric cancer that is one of the most complex forms of human cancer in which a number of genetic and epigenetic factors contribute to the development of the disease, we came to conclusion that we were unable to identify a particular set of genetic alterations that occurs in every instance or form of the disease. The study included more than 200 prospective and 100 retrospective cases that were subjected to systematic investigation of the loss of heterozigosity of the best-known tumor suppressor genes that normally ensure the integrity of the genome, a number of microsatellite markers that are recognized to reveal genomic instability of a patient, and differential expression of oncogenes such as *EGF, FGF-4, c-met, cripto/CR-1, HRG1b, c-erb3/HER3* and *cyclin E* that are widely recognized to play a role in gastric carcinogenesis. Yes, it is true that the combined genotypes produced in this way and when correlated with clinical and histopathological features were to a large extent in accordance with the findings of other researchers, likewise screening for mutations in particular genes contributed to the knowledge of disease development in particular patient, but in both cases we found a lack of information that could lead to some more general conclusions about any wide principle of the disease. Interestingly, when screening for genetic alterations, precancerous lesions such as chronic obstructive gastritis, gastric ulcer, metaplasia or dysplasia revealed similar patterns of genetic changes as found in developed tumors, although it is known that fewer than half of these cases will turn to malignant growth. The question raised whether are there some 'master genes' whose function is critical to make profit on genetic alterations that already occurred or will occur in the future in order to direct a cell to reproduce incorrectly. Analysis of differential expression of proteins vs normal tissues by two-dimensional difference gel electrophoresis followed by mass spectrometry revealed a number of differentially expressed proteins including oncoproteins that may have an important role in molecular pathogenesis of the disease. The same studies are now also performed on model systems of key biological phenomena possibly involved in general oncogenesis such as tissue regeneration, cell cycle regulation and apoptosis. We believe that combination of data from various proteomes and transcriptomes could in the future contribute to better understanding of key mechanisms of tumor initiation and progression.

# Brain tumour invasion & apoptosis: a future therapeutic role for ganglioside inhibitors?

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Malignant primary brain tumours - predominantly gliomas - are not only rising markedly in incidence within the developed world but carry a uniformly dismal prognosis. While intrinsic brain tumours generally fail to metastasise to distant organs they are characterised by diffuse local invasion of normal brain. Debulking surgery may remove much of the main tumour mass, but cells that have migrated from the major tumour mass remain. Radiotherapy, usually after surgery, plays a role in destroying remaining cycling cells. Migratory, "guerrilla" cells, however, are highly resistant to such radiotherapeutic approaches due to their arrest from the cell cycle. In addition, while many cytotoxic drugs enter the main tumour mass through a disrupted blood brain barrier, migratory cells are protected by a functional and intact blood-brain barrier. Gangliosides are acidic glyco-sphingolipids characterised by the presence of one or more sialic acid residues. In neoplastic tissues simple gangliosides are overexpressed and their role in tumour-tumour cell interactions as well as the interactions between healthy cells and tumour cells is pivotal to tumour growth, development and metastasis/invasion. Simple gangliosides (e.g. GM3 and GD3) modulate neoplastic cell adhesion as well as production of extracellular matrices such as laminin as well as certain degradative enzymes (metalloproteinases). The function of integrins, integral components of the cell-to-cell adhesion process, is also modulated by gangliosides.

A crucial step in ganglioside biosynthesis is the glucosylation of the ceramide moiety that leads to the formation of glucosylceramide, the backbone of gangliosides. This reaction is catalysed by glucosylceramide synthase, inhibition of which by the use of N-butyldeoxynojirimycin (NB-DNJ), and other structurally similar compounds (such as NB-DGJ), result in reduced or abated ganglioside synthesis. We have shown that 4 imino sugars with structural similarities to NB-DGJ act as potent modumators of invasive behaviour in glioma by the use of time-lapse microscopy and Transwell invasion assays, using a highly invasive glioblastoma multiforme culture and a cultured giant cell variant glioblastoma (a tumour which although histologically similar shows a limited degree of infiltrative behaviour, both *in vivo* & *in vitro*).

Interestingly, accumulation of ganglioside GD3 in non-neoplastic mammalian cells can lead to mitochondrial damage, which in turn initiates a chain of events leading to apoptosis. However, in most tumour cells GD3 is acetylated into its 9-O-acetyl-GD3 form (by a specific acetylase enzyme - the sialate-9-O-acetyltrasferase), which does not disrupt the mitochondrial membrane and thus does not cause the cells to undergo apoptosis. We are currently developing a gene therapeutic strategy for gliomas targeting this GD3 atecylation pathway.

# Ras mediated gene expression profile and cell death based therapies during multistage carcinogenesis

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Constitutively active forms of Ras are found in a variety of tumours (1) suggesting an important role for this pathway in cancer. Ras activates the MEK-ERK pathway and activated ERK1/2 translocate to the nucleus where they phosphorylate a variety of targets.

We have developed conditionally mouse and human cell systems of activated V12 mutant Harvey Ras oncogene expression. Here we report for the first time that in the absence of growth factors initial cellular exposure to oncogenic Ras only transiently activated the same pathway in the nucleus by a mechanism which involves the phosphotyrosine phosphatase MKP-1 (2). We have also investigated the impact of transient nuclear MAPK activation on the cell cycle as well as to changes in global gene expression profiles by using high density (microarray) analysis. We have also compared early events in Ras signalling with late nuclear effects of Ras associated with cell transformation (3, 4). The interplay between proliferative and apoptotic signals mediated by Ras are going to be discussed.

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# Transcription factor Ets1 is an effector of protein kinase Cα in cancer cells

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The kinase PKCα and the transcription factor Ets1 are often associated with advanced tumor progression. We have previously shown that a truncated, constitutively active form of PKCα is able to increase the transcriptional activity of exogenous Ets1 in breast cancer cells and to phosphorylate Ets1 *in vitro*. To further elucidate a possible link between PKCα and Ets1 we used RNA interference. We were able to design a PKCα-specific small interfering RNA (siRNA) that strongly suppressed the expression of PKCα in cancer cells. In a variety of cancer cell lines (MDA-MB-231, MDA-MB-435, OsA-CL, SK-Mel, and Jurkat T-cells), the endogenous level of Ets1 protein was strongly downregulated when expression of endogenous PKCα was inhibited. This effect could be rescued by MG-132, a proteasome inhibitor, suggesting that PKCα regulates Ets1 expression on the posttranscriptional level. Extracellular-signal-regulated kinase 1/2 (ERK1/2) is responsive to PKCα and is able to activate Ets1 by phosphorylation. However, downregulation of PKCα had no effect on ERK1/2 activity, nor did the inhibition of ERK1/2 modulate the Ets1 protein level. Instead, inhibition of Ca/calmodulin-dependent kinase II (CaMKII) affected Ets1 expression rendering it independent of PKCα. CaMKII inactivates and destabilizes the Ets1 protein which requires the exon VII of Ets1. A natural Ets1 splicing variant ΔVII-Ets1, lacking the exon VII domain, was less affected when PKCα was suppressed supporting the notion that CaMKII is involved in PKCα-dependent regulation of Ets1. We also found that PKCα-specific or Ets1-specific siRNA have similar biological effects. Both enhanced the sensitivity of MDA-MB-231 cells to the anti-cancer drug mithramycin A and increased the ability of cells to attach to surfaces. Also, similar effects on the expression of Ets1-responsive genes were observed. Our data support a model where PKCα not only maintains Ets1 expression in cancer activity but also uses Ets1 to alter gene expression.

# Comparison of expression of cathepsins B and L and MMP-2 in endothelial cells and in aorta ring model of angiogenesis

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The lysosomal cysteine proteinases cathepsins B and L play an important role in the invasive growth of tumour cells, but their association with angiogenesis has not been studied to great extent. High level of cathepsin B was found in endothelial cells of sprouting capillaries but not in old vessels on breast (1) and brain tumors (2,3). The aim of this study was to determine the possible role of endothelial cell associated cathepsins B and L in induced capillary growth in the aorta ring model of angiogenesis. Rings of rat aorta were cultured in 3D collagen matrix in the presence of serum-free endothelial medium to obtain growth of forms closely resembling native capillaries and to study the role of various proteinases in this process. Proteinases gene expression was also studied in vitro in proliferating primary human dermal microvascular endothelial cells (HMVEC-d) under various conditions, using quantitative RT-PCR technique. Specific and selective inhibitors of cysteine proteinases did not inhibit capillary growth in aorta ring culture. We observed only slight inhibition in the degradation of surrounding collagen in regression phase. In contrast, strong inhibition of capillary growth and collagen degradation was observed by matrix metalloproteinase (MMP) inhibitor BB-94, indicating the importance of endogenous MMP activity in angiogenesis. In support of this finding, we demonstrated a significant increase in endogenous endothelial mRNA of MMP-2, but not of cathepsins B and L, in HMVEC-d culture. However, MMP-2 mRNA expression was increased only when the cells were embedded in collagen, but not when grown on plastic, regardless of the addition of growth factors VEGF or bFGF. Moreover, on plastic the impairment of MMP-2 induction by growth factors was observed. Differentional effect of growth factors implies the cross talk with integrin signalling as a consequence of binding to the matrix, giving the basis of further studies of the effect of matrix composition on the expression of different proteinases during endothelial cell proliferation. This study suggests that endothelial cell - associated cathepsins B and L may not be involved in invasive growth of capillary from existing blood vessels in the used model, and other approaches will be undertaken to show their possible role in angiogenesis. Secondly, the presence of collagen is necessary for increased MMP-2 expression in proliferating endothelial cells in the presence and absence of growth factors.

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# Genotoxicity of cadmium and microcystins is mediated by oxidative stress

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For the chemist, reactive oxygen species (ROS) result from dioxygen chemical characteristics and for the physiologist ROS formation is inevitable consequence of aerobic metabolism. Oxidative stress is resulting from redox state "deregulation" and has been implicated in the pathogenesis of many diseases including cancer. There is increasing evidence that exposure to environmental factors like toxic substances and ionizing and non-ionizing radiation induce oxidative stress. ROS can react with lipids, proteins, and cause oxidative DNA damage which can lead to somatic mutations and cancer.

Cadmium has been classified as a human carcinogen but the mechanisms of its action remain unclear. Using the cell mutation assay with human-hamster hybrid AL cells in which both intragenic and multilocus deletions can be detected, we showed that $CdCl_2$ is a strong mutagen that induces predominantly large deletion mutations. Concurrent treatment of $A_L$ cells with the ROS scavenger dimethyl sulfoxide (DMSO) significantly reduced the number of cadmium-induced mutations, while pre-treatment of cells with buthionine sulfoximine, that depletes intracellular glutathione, increased cytotoxicity and mutagenicity of cadmium, indicating involvement of ROS. Using fluorescent probe (5',6'-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate) we demonstrated that cadmium induced formation of intracellular ROS and we also demonstrated that cadmium induced 8-OHdG adducts, which accumulated with prolonged exposure (1). On the other hand, we showed that cadmium inhibited the removal of hydrogen peroxide induced 8-OHdG adducts, indicating its interference with the repair of oxidative DNA damage. Thus, the carcinogenicity of cadmium can be explained by both, its mutagenic activity, which is mediated by reactive oxygen species induced DNA damage and by its interference with the repair of oxidative DNA damage.

Microcystins are naturally occurring hepatotoxins produced by strains of *Microcystis aeruginosa*. They are involved in promoting primary liver tumors and our previous study (2) showed that they might be also tumor initiators. Several recent studies, including ours, indicate that microcystins produce intracellular formation of reactive oxygen species (ROS), which may contribute significantly to microcystin-induced toxicity as well as carcinogenicity. With the comet assay we demonstrated that MCLR at doses that were not cytotoxic (0.01 - 1 μg/ml), induced dose and time dependent DNA strand breaks in human hepatoma cell line HepG2. Using modified comet assay with purified oxidative lesion specific enzymes EndoIII and Fpg we showed that MCLR induced oxidized pyrimidines and purines. Furthermore, we found that during the prolonged exposure of the cells to MCLR oxidized pyrimidines were effectively repaired by cellular DNA repair mechanisms, while oxidized purines accumulated. The formation of DNA strand breaks and oxidized purines was completely prevented by a superoxide dismutase mimic - TEMPOL, an iron chelator - DFO and a precursor of GSH and intracellular ROS scavenger - NAC, and partly by hydroxyl radical scavengers DMSO and DMTU. These results provide evidence that MCLR cause DNA damage indirectly via ROS formation and the observed accumulation of oxidized purines may increase probability for mutations, potentially leading to cancer initiation. We conclude that various initiators and pathways may lead to ROS formation and consequent DNA damage, leading to cancer initiation.

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# Use of preneoplastic lesion in colon (aberrant crypt foci) and liver (enzyme altered foci) in experimental cancer research for the detection of initiating and tumour promoting factors in the diet and for the detection of chemoprotective food constituents

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Precancerous lesions in the colon and liver consist of morphologically and biochemically altered cells. They can be detected in laboratory rodents (mice and rats) after exposure to chemical carcinogens and are valuable tools for the detection and characterisation of car-cinogens and cancer protective compounds. It is assumed that their formation and inhibition is of human relevance since; aberrant foci in the colon were also detected in man and some findings indicate that the molecular alterations (eg mutations in oncogenes) are similar as those found in rodents. The use of foci models in experimental cancer research has the advantage that these alterations are detectable already several (8-12) weeks after the treatment of the animals and that lower animal numbers (ca 8-10 per experimental group) are required than in long term carcinogenicity studies. In the liver, the most frequently conducted experiments are based on the expression of the placental form of glutathion-S-transferase (G+) in cells which can be detected immunohistochemically. In the colon, aberrant crypt foci are scored after staining with methylene blue. Apart from the number of foci, also their size (area in liver and number of crypts/focus in colon) are important parameters. Notable recent developments are the design of an ex vivo model with primary hepatocytes which enables the detection of individual G+ cells, and the establishment of a method for the identification of ß-catenin accumulating crypts which may be represent specific precancerous lesions. By use of different protocols in foci experiments it is possible to discriminate between initiating and tumour promoting compounds and it was shown that certain dietary constituents (eg. heterocyclic aromatic amines which are formed during cooking of meats, nitrosamines, PAHs) cause formation of foci in hepatic and/or colonic tissue and that a number of factors (hypercaloric nutrition, fats, bile acids) have an impact on the size and development of these lesions. Foci models have been also used successfully for the identification of protective factors in the human diet. We will describe the results of several model studies with heterocyclic aromatic amines and other carcinogens in which putative protective effects of components of the diet eg. *lactobacilli* in fermented foods, coffee constituents and cruciferous vegetables and their constituents were studied.

# Pharmacogenetics of thiopurines: can posology be guided by laboratory data?

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Thiopurine drugs (6-mercaptopurine, 6-thioguanine and azathioprine) are antimetabolites thought to act by means of inhibition of *de novo* purine biosyntesis and of DNA metabolism. These drugs are commonly used for the treatment of leukemia and of different immune disorders, such as inflammatory bowel disease (IBD) and rheumatoid arthritis. The toxicity of the treatment with thiopurines, and the occurrence of bone marrow toxicity in particular, have been related to a genetically determined deficiency of the enzyme thiopurine-S-methyl transferase (TPMT), which is responsible for the metabolic inactivation of the drug. Genetic polymorphism causes a reduced TPMT activity in about 10% of Caucasian subjects, and no measurable activity in 1/300. It has been reported that subjects with reduced TPMT activity have a slightly increased risk of developing thiopurine related side effects, while subjects with no measurable TPMT activity have a very high risk of developing a severe form of bone marrow toxicity caused by the drug.

The preliminary data presently reported were obtained in a cohort of 46 patients with IBD treated at Burlo Garofolo Hospital with azathioprine show that the toxicity of the treatment with azathioprine may be caused also by factors different from TPMT reduced activity.

The variability in the toxicity of 6MP has also been the object of analysis, performed at St.Jude Children's Research Hospital, of the genome of pediatric leukaemia patients, using the high throughput technique of gene expression measurement by microarrays. This technique allows the elucidation of the role of genomics in the cellular response to drug treatment, and may contribute to the knowledge of the mechanism of action of the drug thus leading to possible identification of new genetic determinants of thiopurines toxicity and activity. The results obtained with the limited number of cases examined so far indicate that in treated patients the cytotoxic activity of thiopurines is related to the expression of genes involved in the signalling pathways mediated by a small guanosine triphosphatase (the GTPase Rac1), a critical regulator in mammalian cells.

This study provides direct evidence showing that inhibition of Rac1 signalling by 6MP, reported by others *in vitro*, also occurs in clinical conditions. It also supports the view that factors different from TPMT activity play a role in determining thiopurines toxicity, and that laboratory data might be used to identify patients requiring a reduction in posology to prevent subsequent severe toxicity.

# Calcitriol down-regulates αvß$_3$ integrin expression in a mouse Lewis lung carcinoma (LLC) and WEHI-3 leukemia cell lines

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Calcitriol (CAL) [1,25-$(OH)_2D_3$], active hormonal form of vitamin $D_3$, reveals antitumor activity both *in vitro* and *in vivo.* CAL may activate the ß3-integrin subunit gene and promote the plasma membrane appearance of αvß$_3$ on osteoclast precursors and on HL-60 leukemia cells. In this study we evaluated the effect of calcitriol and its new analogue 1,24-$(OH)_2D_3$ (1,24-$D_3$), synthesized in order to avoid hypercalcemic effect of calcitriol, on αvß$_3$ integrin-dependent mouse leukemia and lung cancer invasive properties.

The influence of CAL or 1,24-$D_3$ on the proliferation, adhesion properties and cell cycle were tested by FACS analysis, adhesion and antiproliferative assays. Monoclonal antibodies and flow cytometry were applied for evaluation of integrins expression. Antitumor activity *in vivo* was examined in the model of subcutaneously growing tumors (LLC) in C57Bl/6 mice.

We have shown, that LLC and WEHI-3 cells express a high level of αvß$_3$ (but not of αIIbvß$_3$) integrin and this expression was significantly reduced by the *in vitro* treatment with CAL or 1,24-$D_3$. Both compounds inhibited these cells proliferation and induced apoptosis after 72 or 96h of *in vitro* treatment. Moreover, both agents diminished adhesion of LLC and WEHI-3 cells to fibrinogen (ligand for ß3 integrin subunits). Further, in vitro incubation of LLC cells with both agents retarded the growth of subcutaneous tumors in mice. In all experiments, biological activity of 1,24-$D_3$ was similar or higher than this of CAL, what is in accordance with our previous experiments, showing higher antiproliferative, lower calcemic activities and lower lethal toxicity of new analog as compared to parental drug. Our data demonstrate that calcitriol or its analog 1,24-$D_3$, is a good candidate for an antitumor drug, especially in the treatment of cancers in which the invasive potential is, at least in part, dependent on expression of ß3 integrins. Further studies are undertaken to confirm the hypothesis concerning the involvement of αvß$_3$ integrin in the mechanism of tumor growth inhibition by calcitriol and its new analog.

# Development of a quantitative RT-PCR assay for the urokinase receptor splice variant uPAR Del5

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The receptor for urokinase, uPAR (CD 87) plays an important role in tumor invasion and metastasis by focussing the serine protease urokinase to the tumor cell surface. uPAR is a highly glycosylated, glycan lipid (GPI-)-anchored membrane protein, consisting of three homologous domains (DI, DII, DIII). Each individual domain is encoded by two exons: DI by exons 2+3, DII by exons 4+5, and DIII by exons 6+7. Apart from uPAR wild type (uPAR-wt), two splice variants have been described: uPAR-del5 (lacking exon 5) and uPAR-del4+5 (lacking exons 4 and 5). In the case of uPAR-del4+5 (but not uPAR-wt), we recently presented evidence that higher mRNA expression in tumor tissue is significantly associated with shorter disease-free survival of breast cancer patients (Luther *et al. 2003*, Thromb Haemost 89, 705-17).

In the present study, we established a specific, highly sensitive RT-PCR assay based on the LightCycler technology for detection of the uPAR splice variant uPAR-del5. One of the amplification primers is directed to the alternative splice site of exons 4 and 6, which, therefore, only allows for amplification of uPAR-del5 but not for uPAR-wt or uPAR-del4+5 mRNA. Furthermore, we used exon-spanning primers to prevent amplification of genomic DNA. For quantification of mRNA expression, glass capillaries coated with defined copy numbers of uPAR-del5 plasmid were used, ranging from 100,000 to 10 molecules. The data were normalized against expression of the housekeeping gene G6PDH. In addition to the uPAR-del5 assay, we also developed a novel uPAR-wt assay, which does not detect the alternatively spliced uPAR mRNA variants del5 or del4+5. With these assays, we performed expression analysis of uPAR mRNA in malignant and benign cell lines of different origin (breast, ovary, skin). Both uPAR-wt and the splice variant were detected in these cell lines. RT-PCR results were compared to uPAR antigen levels determined by the uPAR-ELISA assay HU/HD13 (Kotzsch et al. 2000, Int J Oncol 17, 827-34). High (low) uPAR antigen levels correlated with high (low) uPAR mRNA expression (both uPAR-wt and uPAR-del5). Currently, we study uPAR-del5 in comparison to uPAR-wt and -del4+5 expression in a well characterized set of breast cancer tissues.

# Proliferation index and telomerase activity in brain tumours

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**Objectives:** To find a correlation between the proliferation index and telomerase activity in brain tumours.

**Materials and methods:** Tissue specimen from 47 patients with brain tumours were collected during open surgery and partly stored in liquid nitrogen, fixed in 4% buffered formalin or grown in cell culture flasks as a cell culture.

Telomerase activity was determined using the TRAPeze Telomerase Detection Kit (Intergen).

Proliferation activity was immunohistochemically detected with a monoclonal antibody against Ki-67 (clone MIB-1, Dako).

Telomerase protein (catalytic subunit, hTERT) was immunohistochemically detected with a monoclonal antibody against hTERT (Human Telomerase Reverse Transcriptase, clone 44F12, Novocastra).

**Results**: All tumour biopsies were positive for the proliferation marker but percentages varied depending on malignancy of the tumours. All malignant biopsies expressed hTERT but only 36% of benign meningiomas were positive in very low percentages. Telomerase activity was found in 60 % of malignant brain tumour biopsies and in only 15 % of benign meningiomas. There was a detectable telomerase activity in 37% of cell cultures derived from benign meningiomas.

**Discussion:** It seems that telomerase activity can be detected in brain tumours with a high proliferation index and that malignant TRAP-positive tumours have a higher proliferation index than TRAP-negative. Benign meningiomas expressed almost no telomerase activity and no or low levels of hTERT, as a biopsy. Benign meningiomas, negative as a biopsy, seem to have potential to express telomerase activity in a primary cell culture.

| TUMOUR | NUMBER | BIOPSY | | | | PRIMARY CELL CULTURE | |
|---|---|---|---|---|---|---|---|
| | | MEAN % Ki-67 immunostaining (min-max) | MEAN % hTERT immunostaining (min-max) | TELOMERASE ACTIVITY (TRAP PCR) | | TELOMERASE ACTIVITY (TRAP PCR) | |
| | | | | NUMBER ANALYSED | POSITIVIY (%) | NUMBER ANALYSED | POSITIVIY (%) |
| MENINGEAL TUMOURS | 34 | 2.3 (0.2 - 18.6) | 7.5 (0.0 - 64.5) | | | | |
| Benign | 30 | 1.6 (0.2 - 5.3) | 3.7 (0.0 - 29.6) | 20 | 3 (15%) | 16 | 6 (37.5%) |
| Malignant | 4 | 7.7 (1.9 - 18.6) | 35.7 (0.6 - 64.5) | 4 | 2 (50%) | 1 | 1 (100%) |
| GLIALTUMOURS | 10 | 7.6 (0,1 - 20,2) | 20.8 (0.0 - 80.6) | | | | |
| Astrocytoma LG | 4 | 1.7 (0.1 - 4.6) | 0.0 | 2 | 0 (0%) | 2 | 2 (100%) |
| Astrocytoma HG | 1 | 4.5 | 2.6 | 1 | 0 (0%) | 1 | 1 (100%) |
| Glyoblastoma multiforme | 5 | 13 (2.1 - 20.2) | 41.0 (75.8 - 93.2) | 4 | 2 (50%) | - | - |
| PNET | | | | | | | |
| Medulloblastoma | 3 | 26.0 (17.5 - 40.3) | 85.6 (75.8 - 93.2) | 2 | 2 (100%) | 1 | 1 (100%) |

# Role of lysosomal cysteine peptidases in tumour progression

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Proteolysis in close vicinity of tumour cells is a hallmark of cancer invasion and metastasis. Since human and murine genomes encode more than 400 proteases each, it is of high interest to identify the proteases that are critically important for tumour progression *in vivo*. To investigate the role of the lysosomal cysteine peptidase cathepsin B (CTSB) in tumour progression and metastasis, *ctsb*-deficient mice were crossed with a mammary cancer-susceptible mouse strain (MMTV-PyMT). Tumour-prone mice heterozygous or deficient for CTSB showed an identical onset of tumour formation as compared to CTSB wild-type mice, but the size of primary carcinomas and lung metastases was reduced. When primary PyMT tumour cells of different CTSB-genotypes were intravenously injected into congenic females of the three CTSB-genotypes, the size of lung tumour colonies was largely determined by CTSB of stroma cells, whereas tumour cell CTSB had only a minor effect. Co-localization of CTSB and tumour infiltrating macrophages revealed that cathepsin B expressed by these inflammatory stroma cells contributes significantly to *in vivo* growth of the tumours. Thus, our results provide *in vivo* experimental evidence for the connection of cathepsin B, tumour associated inflammatory cells and cancer progression.

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# Endothelial cells: impact on malignancy of brain tumors

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**Background:** Malignant gliomas are common brain tumors bearing a bad prognosis despite intense therapeutical effort. They typically present a markedly increased angiogenesis, which is dependent from tumor grade. To evaluate basics of glioma vascularization, research efforts using organotypic endothelial cells (EC) as well as tumor derived EC are mandatory.

**Methods:** Microvascular ECs were isolated and cultured from healthy brain tissue and from gliomas WHO grade I-IV by the modified method of Bowman *et al.* (1982). Fresh tissue from the operation room was minced and digested enzymatically, followed by percoll gradient centrifugation. Human brain derived EC were then separated using magnetic beads linked to UEAI, CD105-, CD31-, E-selectin- and VE-cadherin antibodies. Positive cells were seeded into culture flasks coated with gelatine. Cells were characterized immunohistochemically by the EC specific markers vWF, GLUT 1 and by their uptake of DiI-Ac-LDL. Proliferation was quantified by the MTT test. MMP expression was analyzed by PCR. As a functional assay we used the tube formation assay on Matrigel.

**Results:** EC morphology did not correlate with tumor grade. EC from high grade gliomas showed significantly higher proliferation rates than ECs from low grade gliomas and ECs from healthy brain tissue. Only EC from high grade gliomas expressed MMP-7 and MMP-9 on RNA level. No difference was observed in expression of MMP 2 and

**3. Conclusions:** A reproducible method for the isolation of EC from brain tumors could be established successfully. First data indicate "malignant" properties of ECs isolated from high grade gliomas, compared to low grade glioma or normal brain EC. Therefore, endothelial cells seem to be a significant cause of malignant properties of high grade gliomas.

# Angiogenesis independent brain tumour growth

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By xenotransplantation of human brain tumours into immuno-deficient nude rats, highly infiltrative tumor phenotypes were esatblished that coopted the host vasculature and mediated an aggressive disease course without signs of angiogenesis. The malignant cells expressed neural stem cell markers and showed a migratory behavior similar to normal human neural stem cells. The tumor stem cells displayed a self-renewal capacity and gave rise to tumors *in vivo*. By serial animal passages, the stem cell tumours gradually aquired angiogenesis dependency. This process was accompanied by a reduction in stem cells markers. Gene arrays revealed an up-regulation of numerous pro-invasive genes in the stem cell tumors, whereas genes involved in angiogenesis signaling pathways were down-regulated . In contrast, the angiogenesis dependent tumors, derived from the stem cell tumors, showed a down-regulation of pro-invasive genes. The described angiogenesis independent tumor growth and the uncoupling of invasion and angiogenesis, represented by the cancer stem cells and the cells derived from them respectively, points at two completely independent mechanisms that drive tumor progression. Array comparative genomic hybridization revealed little structural differences between the stem cell tumors and the angiogenesis dependent tumors derived from them. This indicates that the phenotypic shift is on the transcriptional level. The presented work underlines the need for developing therapies that specifically target the cancer stem cell pools in tumors.

# Cystatin C in non-Hodgkin lymphoma patients: possible relation to anti-tumour immune response

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Dendritic cells (DC) process and present antigen via MHC II pathway, which results in the initiation and development of antigen-specific and $CD4^+$ T cell-mediated immune response. Furthermore, cross-presentation by DC, i.e. presentation of exogenous antigen by MHC I to $CD8^+$ T cells, is thought to play a critical role in direct triggering of anti-tumour immune response. An inhibitor cystatin C was suggested to interfere with the antigen loading process to MHC II in DC. In this study cystatin C content, localization and extracellular secretion were followed during human dendritic cell differentiation and maturation *in vitro*. Furthermore, lymph-node tissue of non-Hodgkin lymphoma patients was compared to non-infiltrated lymph nodes for the presence of DC, highly immunolabeled with anti-cystatin C monoclonal antibody.

Our results of in vitro studies with DC demonstrate differentiation and maturation dependent cystatin C content, localization and extracellular secretion. According to the different localization of cystatin C in immature and mature DC, we demonstrated the presence of large population of mature DC in non-infiltrated lymph nodes, compared to diminished DC population, mostly of immature maturation stage, present in infiltrated lymph nodes of non-Hodgkin lymphoma patients. We suggest cystatin C intracellular localization might be used as a marker for the maturation stage of DC inside infiltrated and non-infiltrated lymph node tissue.

# Angiogenesis and therapeutic targeting of the tumour vasculature

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The tumour vasculature is an attractive target for therapy because of its accessibility to blood-borne anti-cancer agents, the reliance of most tumour cells on an intact vascular supply for their survival and its role as a major route for metastatic spread. Therapeutic vascular targeting can be divided into *anti-angiogenic* approaches, which aim to disrupt the processes involved in the out-growth of new blood vessels from pre-existing ones and *anti-vascular* approaches, which aim to target the established tumour vasculature, rapidly shutting down blood flow. There are numerous anti-angiogenic agents in clinical trial for cancer, mostly targeting the early steps in tumour angiogenesis, namely breakdown of the vascular basement membrane and tumour matrix, production/activity of pro-angiogenic growth factors, particularly vascular endothelial growth factor (VEGF), and migration/proliferation of vascular endothelial cells. Further advances in this area are likely to arise from increased understanding of tumour neovascularization via the processes of intussusceptive luminal splitting of blood vessels, vasculogenic mimicry, vessel co-option and recruitment of circulating endothelial progenitor cells.

The search for new therapeutic targets on *established* tumour blood vessels is an expanding field. Methods used include powerful bioinformatic analyses of published data and comparative analysis of gene expression on endothelial cells isolated from tumours and corresponding normal tissue. Several putative targets have been identified including the vascular developmental gene Robo4 (Huminiecki *et al.*, 2002, Genomics 79:547-552) and the Tumour Endothelial Markers (TEMs) (St Croix *et al.*, 2000, Science 289:1197-1202). Therapeutic approaches include targeting with antibodies, antibody fragments or peptides. Radio-isotopes, cytotoxic drugs and tissue factor for inducing coagulation have been conjugated to antibodies as vascular damaging strategies.

The colchicine-related tubulin-binding/microtubule-depolymerizing agents such as the combretastatins are the biggest low molecular weight group of drugs currently in clinical trial as tumour anti-vascular agents (Tozer *et al.*, 2002, Int J Exp Pathol 83:21-38). The potency of these agents against tumour blood vessels was identified from a small *in vivo* screening exercise, which highlighted the endothelial cytoskeleton as a potential target for further drug development. Much is now known about the mechanism of action of the combretastatins at the levels of the endothelial cytoskeleton and intact tumour blood vessels. However, the characteristics of tumour blood vessels, which make them uniquely susceptible to these agents, are still poorly understood. The development of tumour lines, which are genetically modified to produce different levels/types of growth factors involved in the maturation process of newly formed blood vessels should provide a route towards clarifying these issues. Results will be important for providing leads to further drug development in this area.

# Development of potential anti-cancer agents: diazenes and derivatives

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In order to improve the effectiveness of cancer treatment, new anticancer drugs have been synthesised that may reduce the activity of some of the essential molecules for cellular homeostasis. Glutathione (GSH), a ubiquitous thiol containing tripeptide, is recognized to be such target. Its role in the cellular defence due to detoxification of xenobiotics, and protection against oxidative stress has been recognised many years ago and has been studied for years. Recently, however, evidence has emerged that suggest the essential role of GSH in the activation or inhibition of proteins fundamental to cell survival, especially in the control of apoptotic cell death. These findings focus the research to the discovery of drugs that may be GSH targeted, thus inhibiting the growth of tumour cells.

We have synthesised very recently new compounds, diazenes, as selective oxidants for a mild transformation of various thiols to the corresponding disulfides (1). Diazenes easily oxidize cysteamine, thiosalicylic acid derivatives, dithiothreitol and others in methanol at room temperature. Under quasi-physiological conditions they interact also with GSH (1). That stimulated us to examine their cytotoxic activity against several tumour cell lines *in vitro*.

This presentation is the review of five years research collaboration between Slovenian group that synthesises new compounds, diazenes and their derivatives (team leader S. Polanc), and Croatian group that examine the cytotoxicity of these compounds and the causes of cytotocixity (team leader M. Osmak). So far we have examined about sixty new compaunds. We have found that: a) several diazenes inhibited the growth of different tumour cell lines (glioblastoma, cervical, laryngeal and mammary carcinoma cells) (2-8); b) some of them reduced the survival of drug-resistant cells as well (the ability of malignant cells to develop resistance to anticancer drugs is the major obstacle to the ultimate success in cancer therapy) (2, 5-7); c) for some of these compounds the target was the intracellular glutathione (3, 6); and d) several diazenes were able to revert the resistance of tumour cells to cisplatin and /or vincristine (3,4). For the most promising new compound, the type of induced cell death was examined as well. The most important findings of this research will be presented in more details.

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# COX-2, a new target for cancer prevention and treatment

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Metabolites of arachidonic acid participate in normal growth responses as well as in aberrant growth and proliferation, including carcinogenesis. The key step in the conversion of free arachidonic acid to prostaglandins (PGs) is catalysed by the rate-limiting cyclooxygenase enzyme (COX). In vertebrates, two isoforms of COX have been identified. COX-1 is expressed constitutively and ubiquitously and is thought to generate PGs that control normal physiological function such as maintenance of the gastric mucosa and normal platelet function. On the other hand, COX-2 is undetectable in most tissues except brain, pancreatic islets, ovary, uterus and kidney, but becomes strongly induced upon hormonal stimulation or environmental stress, for example in the course of inflammatory processes and tissue repair. An enhanced expression of COX-2 is observed frequently in advanced carcinomas, which in turn suggests that enhanced arachidonate metabolism may play a crucial role in tumour biology. COX-2 is over-expressed in cancerous lesions in many cancers and the degree of its expression has been related to survival. COX-2 is also over-expressed in the precancerous lesions. The mechanism by which COX-2 is thought to be involved in the carcinogenesis include resisting apoptosis, increasing cell proliferation, stimulating angiogenesis and modulating the invasive properties of cancer cells.

Population-based studies have established that long-term intake of non-steroidal anti-inflammatory drugs (NSAIDs), compounds that inhibit the enzymatic activity of COX, reduce the relative risk for developing cancer. COX-2 represents the major cellular targets of non-selected COX inhibitors and the COX-2 selective inhibitors "coxibs". A second-generation of COX-2 inhibitors with less associated toxicity to gastric mucosa has been developed. Recent studies in humans indicate that therapy with specific COX-2 inhibitors might be an effective approach to cancer prevention and therapy. An exciting area for future investigation will be to test whether combined treatment with COX-2 inhibitors and drugs that target other oncogenic pathways can lead to improved clinical outcome.

# Clomipramine (Anafranil™) as an anti-neoplastic agent in the clinical management of patients with malignant intrinsic brain tumours

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A case-matched control study has been set up to determine the efficacy of clomipramine as an adjuvant therapy in the treatment of patients with WHO grade III anaplastic astrocytoma and WHO grade IV glioblastoma multiforme brain tumours. The primary outcome measures of the trial are survival time, tumour regression/radiological response. Patients undergoing stereotactic biopsy or tumour resection at King's College Hospital, London are consented into the study. Approximately 130 patients will be recruited in total (and a corresponding 130 case matches will be made.) Patients' aged 16-65, presenting with a primary brain tumour, without other life threatening illness are eligible to take part. Contraindications include use of MAOIs, a pre-existing heart condition, and concurrent chemotherapy. Special consideration will be given prior to recruitment of patients with a history of epilepsy, kidney or liver problems, glaucoma, thyroid disease or prostate complications.A small amount of the brain tumour removed in surgery is used to set-up primary cell cultures (details of this are also presented at this conference). Blood samples from the patients are used to determine the CYP2D6 and CYP2C19 genotype of the patients and also to monitor the plasma concentrations of clomipramine during the trial. Patients begin taking clomipramine as soon as possible after diagnosis; a dose escalation over 4 weeks concludes with patients taking 150 mg/day.

The effect of survival time will be measured using the Kaplan-Meier statistical method. The impact of variables such as age, sex, tumour type, tumour location and genotype on the efficacy of clomipramine will be assessed using log-rank multivariate analyses.

The Samantha Dickson Research Trust sponsors this work.

# Cysteine proteases, cathepsin B and cathepsin L, in glioma progression - a neurosurgeon's aspect

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**Background**. Local invasive growth is a key feature of primary malignant human brain tumours. The proteases, such as lysosomal cathepsins, play an important role during the invasion process. Cysteine cathepsins B and L and their endogenous, intracellular inhibitors, stefins A and B, were implicated in the progression of various types of tumours. Therefore, the knowledge on their involvement in brain tumour development would improve the clinical application of cathepsins in prognosis and could contribute to the development of therapeutically useful agents.

**Patients and methods**. In the retrospective clinical study, the stored histological slides of hundred (100) randomly selected patients were reviewed. Representative sections were treated with monoclonal antibodies against cathepsins (Cat) B, L and stefins A and B. We evaluated the frequency and the intensity of staining in tumour and endothelial cells, scored from 0-12. The statistical analysis comprised descriptive methods and survival analysis.

**Results.** The patients included 52 men and 48 women, aged from 7-75 years, median value 54 years. Many suffered from malignant tumour (73 out of 100), including 58 glioblastoma multiforme. The analysis included 100 cases of primary brain tumour and 11 cases from reoperation. Stefin A and stefin B immunostaining was weak and detected only in a few tumours. Cat B stained positive in almost all cases (98.2 %), while Cat L stained in 87.7 %. The score in tumour and endothelial cells was significantly higher in malignant tumours compared with benign tumours for Cat B ($p < 0.000$) and Cat L ($p = 0.005$). Imunohistochemical (IHC) score of Cat B and Cat L in tumour cells in benign compared to malignant tumours was significantly different ($p = 0.008$ and $p = 0.001$ respectively). IHC score in endothelial cells was significantly higher in malignant tumours for Cat B ($p < 0.000$), but not for Cat L. IHC localization of Cat L showed intense immunoreactivity in tumour cells, while endothelial cells in the tumour tissues were almost devoid of cathepsin L immunoreactivity. A significant correlation was observed between overall staining of Cat B and Cat L ($p = 0.000$). The significant correlation between immunostaining of both Cats (scored together for tumour and endothelial cells) and histological score was also demonstrated ($p = 0.000$ for Cat B and $p = 0.004$ for Cat L). Univariate survival analysis indicated that total Cat B score above 8 was a significant predictor for shorter overall survival ($p = 0.02$). The survival analysis did not confirm the prognostic role of Cat L. In glioblastoma multiforme, intense Cat B staining of endothelial cells was a significant predictor for shorter survival ($p = 0.03$).

**Conclusions**. Cat B expression is of similar intensity in malignant glioma cells and in endothelial cells of primary tumours of central nervous system, while Cat L is localized mostly in tumour cells. The results show that Cat B and Cat L activity participates in local brain tumour invasion. However, whereas the level of expression of Cat B in tumour and endothelial cells is prognostic for the survival rate of brain tumour patients, CatL was not. Cat B in endothelial cells alone is a good prognostic marker for glioblastoma, indicating that Cat B is also playing a role in angiogenesis. Cat L could only be used as biological marker for local invasion and malignancy of primary brain tumours, along with CatB, although they have different additional functions in brain tumour progression. We think that considering Cat B and Cat L immunostaining could improve decision making in daily clinical practise.

# Tumor markers in clinical oncology

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The subtle differences between normal and tumor cells are exploited in the detection and treatment of cancer. These differences are designated as tumor markers and can be either qualitative or quantitative in their nature. That means that both the structures that are produced by tumor cells as well as the structures that are produced in excessive amounts by host tissues under the influence of tumor cells can function as tumor markers. Speaking in general, the tumor markers are thus specific molecules appearing in blood or tissues and the occurrence of which is associated with cancer.

According to their application, tumor markers can be roughly divided as markers in clinical oncology and markers in pathology. In this review, only tumor markers in clinical oncology are going to be discussed. Current tumor markers in clinical oncology include (i) oncofetal antigens, (ii) placental proteins, (iii) hormones, (iv) enzymes, (v) tumor-associated antigens, (vi) special serum proteins, (vii) catecholamine metabolites, and (viii) other molecular species.

As to the literature, an ideal tumor marker should fulfil certain criteria - when using it as a test for detection of cancer disease: 1) positive results should occur in the early stages of the disease, 2) positive results should occur only in patients with a specific type of malignancy, 3) positive results should occur in all patients with the same malignancy, 4) the measured values should correlate with the stage of the disease, 5) the measured values should correlate to the response to treatment, 6) the marker should be easy to measure. Most tumor markers available today meet several, but not all criteria. As a consequence of that, some criteria were chosen for the validation and proper selection of the most appropriate marker in a particular malignancy, and these are: 1) markers' sensitivity, 2) specificity and 3) predictive values. Sensitivity expresses the mean probability of determining an elevated tumor marker level (over the "cut off value") in a tumor-bearing patient. Specificity expresses the mean probability that a normal tumor marker value derives from a tumor-free individual. The predictive value shows the applicability of a tumor marker in a mixed group of patients.

Many theoretical applications exist for tumor markers in clinical oncology. Clinically important utilization of markers includes (i) early detection of the tumor, (ii) differentiating benign from malignant conditions, (iii) evaluating the extent of the disease, (iv) monitoring the response of the tumor to therapy and (v) predicting or detecting the recurrence of the tumor. Since no ideal tumor markers with adequate sensitivity and specificity currently exist, they are only exceptionally used for the screening (prostate specific antigen - PSA). Nevertheless, tumor markers can play a crucial role in detection of an early disease relapse and assessment of response to therapy in selected groups of patients. In monitoring patients for disease recurrence, tumor marker levels should be determined only when there is a potential for meaningful treatment.

# Clinical utility of serine proteases in breast cancer

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Tremendous improvements in the treatment of breast cancer have been made during the last decades; due to early detection and proper adjuvant systemic therapy, the survival rates improved from around 50% to more than 70% in operable disease. According to the meta-analysis in which the data obtained from more than 50000 patients participating into different studies were included, adjuvant systemic therapy was found to reduce the risk of death by approx. one third in all operable breast cancer patients, irrespective of their individual risk of the death based on the traditional prognosticators, such as lymph node involvement, tumor stage, tumor grade, etc. According to this knowledge more and more patients are offered systemic adjuvant therapy all over the world and the knowledge about the pure prognostic biological markers of the disease is not of such interest anymore. However, there is a continuous search for new biological markers according to which we could better predict the response of each individual patient to the particular therapy and serine protease are among them.

The independent prognostic value of serine protease urokinase-type plasminogen activator uPA and its inhibitor PAI-1 in breast cancer has been almost uniformly confirmed in numerous individual studies as well as in a meta-analysis, including 18 data sets of more than 8000 patients. According to these observations, the risk of relapse in node negative patients with low levels of uPA and PAI-1 is less then 10%, and these patients could be spared from toxic adjuvant systemic therapy. Clinically even more relevant and important is the information from the Munich and Rotterdam group that high levels of PAI-1 alone or in combination with uPA may predict for a better response to adjuvant chemotherapy. According to the available data serine proteases may also predict for response to hormonal therapy, although the data on this are not uniform. According to our data obtained on the collective of 460 operable breast cancer patients, high levels of uPA and PAI-1 in primary tumor may be predictive for a better response to hormone therapy and not to chemotherapy. In our study high uPA and/or PAI-1 levels were found to be significantly associated with a higher risk of relapse in patients without any adjuvant systemic therapy and in patients treated with adjuvant chemotherapy (HR 2.14 and HR 2.55, respectively). However, in patients treated with adjuvant hormonal therapy, either alone or in combination with chemotherapy, the prognostic value of uPA and PAI-1 was diminished; even more, high levels of both uPA and/or PAI-1 were associated with a lower risk of relapse (HR 0.30 and HR 0.54, respectively; both:non-significant). On the basis of currently available evidence, serine protease uPA and its inhibitor PAI-1 are certainly the markers that improve proper selection of candidates for adjuvant systemic therapy and maybe also the markers that could improve treatment decision in each individual patient, which is of utmost importance.

# Establishment and characterization of 7 new TIMP-1 monoclonal antibodies

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**Background:** Matrix metalloproteinases (MMP's) are involved in cancer cell invasion and metastasis. One of the natural occurring inhibitors of MMP's is Tissue Inhibitor of Metalloproteinases-1 (TIMP-1), which is a 28.5 kDa glycoprotein consisting of 184 amino acids in the mature protein. TIMP-1 has several other functions in the body, such as, stimulation of cell growth, inhibition of apoptosis, and regulation of angiogenesis. Immunological quantitation of total TIMP-1 has shown strong association to patient prognosis in several types of cancer.

**Aim:** Develop and characterize new monoclonal antibodies (mAbs) in order to optimize TIMP-1 measurements in specimens from cancer patients.

**Methods:** Recombinant human TIMP-1 was used to raise mAbs in mice. An ELISA assay was used to reveal linear epitopes recognized by the mAbs. The ELISA plates contained 15 amino acid long peptides (Mimotopes Pty Ltd., UK), which covered the entire human TIMP-1 sequence. Surface plasmon resonance (BIAcore) was used to test the affinity of the mAbs for TIMP-1 and for their ability to sandwich with each other and TIMP-1. All antibodies were tested in both the capture and detection positions in the sandwich experiments.

**Results:** All 7 antibodies bound to recombinant TIMP-1 coated microtiter wells. Six of the seven new TIMP-1 antibodies are IgGs. Antibody VT3 belongs to the IgA subclass. Antibody VT6 recognizes a linear epitope in the middle of the TIMP-1 sequence very strongly and antibody VT7 binds moderately to a linear epitope in the C-terminus of TIMP-1. VT2 recognizes a linear epitope at the N-terminus of the TIMP-1 protein. The affinity of the monoclonal antibodies is in the range of 6.6*108 to $2.3*10^{13}$ 1/M. VT4 and VT5 have the greatest affinity for TIMP-1. The mAbs demonstrate different binding ability in the capture position compared to the detection position. VT2 performs well as a capture and detection antibody. VT1, VT4 and VT5 perform best as detection antibodies. VT6 and VT7 do not sandwich well with any of the other antibodies. VT6 and VT7 bind strongly to TIMP-1 antigen, which has been coated onto a microtiter plate or onto a membrane (Western blot).

**Conclusion:** These experimental results indicate that the 7 new TIMP-1 antibodies are unique antibodies and may be useful in designing a broad range of different types of TIMP-1 assays.

# Cathepsins and their inhibitors as tumor markers in head and neck cancer

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Cathepsins are ubiquitous lysosomal proteolytic enzymes. As their chemical composition, cathepsins are glycoproteins, majority of them belonging to the group of endopeptidases, i.e. of cysteine and aspartic classes. Endogenous, i.e. physiological inhibitors of cathepsins naturally present in tissues, constitute a single protein superfamily of cystatins. This is subdivided into three families: stefins, cystatins and kininogens.

During the last decades it has been shown that pericellular proteolysis has multiple roles in virtually all aspects of the normal life of a cell. The same process is involved in the degradation of extracellular matrix barriers during invasion and metastasizing. It is believed that proteases can act as tumor progression factor in all stages of malignant progression. They can create a local environment that is conductive to the growth, survival, and progression of a tumor through the modulation of growth factor pathways, cell-cell adhesions, and cell-matrix adhesions.

The contribution of extracellular proteolysis to the progression of carcinoma of the breast, lung, and colorectum has been studied extensively, on preclinical level and in the context of clinical trials. The role of individual proteolytic enzymes and inhibitors as markers predicting the response to various treatment regimens and/or the prognosis of patients in term of various survival endpoints was widely investigated. On the basis of the data available from literature, the general conclusions would be as follows: (1) numerous proteolytic enzymes and inhibitors of various classes are implicated in the invasive behavior of cancer cells; (2) the variations in regulation of proteolytic pathways seem to be the inherent characteristic of individual cancer type; (3) potential role as predictive or prognostic markers was strongly suggested for some of these proteins and even recommended to be used in routine clinical practice; and (4) the results are critically dependent on methodological approach used for enzyme and inhibitor determination in biological samples.

Compared to the previously mentioned tumors, the squamous cell carcinomas of the head and neck fall into much less investigated group of cancers. There are several reasons, the two most important being: (1) head and neck carcinomas appear to be a heterogeneous group of tumors consisting of multiple primary sites inside the upper aerodigestive tract, each with its own natural history and treatment outcome; and (2) the incidence of head and neck carcinomas is much lower compared to breast, lung or colorectal cancer. Consequently, it is very difficult to conduct a study with sufficient number of patients included to obtain statistically meaningful results. It is, therefore, little surprise that no factor within the wide spectrum of biochemical and histological factors has yet been identified as reliably predicting the natural course of the disease or its response to therapy.

The aim of the present study was to present and evaluate the data collected from the existing literature on cathepsins and their inhibitors as tumor markers in head and neck cancer. The results of our own investigations on this topic will be discussed and in particular the applicability for routine clinical practice will be considered.

# Plasma levels of tissue inhibitor of metalloproteinases 1 measured during follow-up of colorectal cancer patients have clinical value in predicting patient outcome

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**Purpose:** We have previously demonstrated that high preoperative plasma levels of Tissue Inhibitor of Metalloproteinases 1 (TIMP-1) predict short survival in patients with colorectal cancer. In the present study we extended our investigations to TIMP-1 measurement of plasma samples obtained 6 months following primary surgery for colorectal cancer.

**Methods**: 296 colorectal cancer patients were included in the study. 47 patients had Dukes' A disease, 136 Dukes' B and 113 Dukes' C. All patients received intended curative surgery but no adjuvant chemotherapy was administered. Corresponding plasma and serum samples were obtained preoperatively and 6 months postoperatively. The median follow-up time was 82 months (range 68 to 95); 144 patients died, 64 patients experienced local recurrence and 51 patients distant metastases during the observation period. Plasma TIMP-1 and serum CEA were measured by ELISA in all samples.

**Results:** Preoperative and postoperative plasma TIMP-1 levels were scored as low or high based on the 95th percentile of plasma TIMP-1 in an age matched healthy control group. 87% (169/193) of the patients with low preoperative TIMP-1 levels remained low postoperatively (group I) and accordingly 12% (24/193) progressed to high postoperative TIMP-1 levels (group III). 59% (61/103) of the patients with high preoperative levels had low postoperative TIMP-1 (group II) and accordingly 41% (42/103) remained high 6 months following surgery (group IV). Analysis of overall survival showed a significant difference between groups ($p=0.002$). With group I as the baseline, group II had a hazard ratio (HR) of 0.9 (95% CI: 0.6 to 1.4), group III 2.1 (1.2 to 3.6) and group IV 1.9 (1.2 to 2.9). Multivariate analysis including Dukes' stage, age, gender, tumor location, and plasma TIMP-1 showed that plasma TIMP-1 was a significant ($p=0.005$) predictor of survival. Similar results were obtained for time to local recurrence and detection of distant metastases. Plasma TIMP-1 remained significant in the multivariate model including serum CEA ($n=285$). Separate analyses including only the 6 month value of TIMP-1 indicated that elevated levels were associated with shorter survival ($p<0.0001$) entered as a continuous covariate. Conclusion: This study suggests that plasma TIMP-1 levels measured during follow-up of colorectal cancer patients after curative surgery have clinical value in predicting patient outcome.

# *Ex vivo* flow cell electropulsation

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**1- What electropulsation can do to cells (fusion, loading, transfection)?** When a short lived electric field pulse is applied to a cell, the resulting change in membrane potential difference may result in a localised change in the membrane organisation. This new state of the membrane is called " electropermeabilized " and can support the transfer of hydrophilic compounds into the cytoplasm and their leakage out of the cell. A key feature is that under controlled electrical conditions this membrane change is transient and the " normal " impermeable state can be recovered. The cell viability can therefore be preserved. This is obtained by a proper choice of electrical parameters (field strength, pulse duration and number of pulses) and buffers (pH, osmotic pressure and additives). This brought the technical possibility to introduce (load) exogenous compounds (drugs) into cells. A clinical development was proposed with big success (electrochemotherapy). This transient membrane organisation remains poorly characterised from a structural point of view. A peculiar associated property is fusogenicity. When cells, which are in the electropermeabilized state, are brought into contact, membrane coalescence occurs leading to the formation of viable polykaryons (electrofusion). This is indicative that the repulsive hydration forces have been abolished. This was supported by the observation of an alteration of the interfacial layer of electropermeabilized cells. A more recent observation is that it was possible to get protein expression by electropulsing cells in a solution containing the relevant plasmid (electrotransformation, electrotransfection). This again can be obtained in vivo (electrogenotherapy).

**2- How to treat a large volume of cells under safe (potentially clinical) conditions?** *Ex vivo* treatment of cells appears as an interesting procedure for cell therapy. A present limit of electropulsation is that most protocols were designed for batch process. Only limited volumes can be treated due to the power limitations of most pulse generators. Furthermore safe conditions avoiding contamination (either microbial or electrochemical) need the use of rather expensive equipments (large laminar flow hood, sterilisation of the cuvettes). Almost 20 years ago, we introduced the concept of follow-through electropulsation. Cells are flowing through a pulsing chamber where they are submitted to a well-defined number of calibrated pulses. A proper setting of pulse frequency and flow rate controls the number of pulses. A large volume of cells can therefore be electrotreated in a small sized pulsing chamber. Power specifications for the pulse generator are mostly driven by the required pulse frequencies. Nevertheless kHz trains can be delivered meaning that high flow rate (i.e. large volumes) can be treated (up to 1 l/s !). Contamination can be avoided by using closed loop circuits.

**3- Physical problems with flow processes.** As with batch processes, Joule heating and electrochemically mediated release from the electrodes must be kept under control. The design of the electrodes must take into account the need for a homogeneous field distribution. Hydrodynamic mechanical stresses are applied on the cells due to the flow. Due to the viscoelastic behaviour of a cell, this results in a change in the cell shape (from a sphere to an elongated spheroid). This would change the cell response to the external field.

**4- Loading blood cells with xenoproducts.** *Ex vivo* blood cell electroloading is easily obtained by the flow process. Due to the cell size heterogeneity, a subpopulation targeting was obtained by a proper choice of the field intensity. White blood cells were electroladed while red blood cells remained unaffected. Hemolysis was therefore avoided. Physiological properties of white blood cells were preserved after their electroloading (ROS generation after contact with opsonized particles, in vivo targeting to organs after inflammation). The flow system can be connected to the patient in a close loop avoiding external contaminations and infection by donor blood.

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**5- Fusion.** Mass electrofusion was obtained by two different approaches: contact first and pulse first. In the first process, adherent cells were grown on microcarriers up to a confluent state. These cellular contacts are known to be well suited for leading to fusion when the cells are electropulsed. The cell covered carriers flow in suspension in the flow through chamber where they are submitted to electric pulses. Fusion was obtained at a rate similar to what was observed on cells growing and pulsed plated. The technical limit was that this process was only suited for attached cells. In the second process, cells in suspension were pulsed in flow and then brought into contact by a gentle centrifugation. Parameters were analogous to what we did in a batch method. High yields of fusion (polykaryon index) were obtained while the cell viability was preserved.

**6- Gene transfer.** Flow electrotransformation is suited for gene transfer. Conditions are similar to what is used for batch approach. Expression was detected at a similar level. Mass formation of transfected cells can be obtained when large quantities of plasmid are available.

# *In vivo* electroporation for effective drug delivery

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Exposure of cells to electric field may change plasma membrane permeability without affecting cells viability or function. In this way molecules which otherwise can not penetrate the membrane, or the membrane represents a considerable barier for them, can enter the cell. Developments in last years proved that this approach can be successfully applied *in vivo* for improving local effectiveness of some chemoterapeutic drugs in treating tumors as well as for gene transfection. The former is referred to as electrochemotherapy and has already entered active clinical research period whereas the latter is referred to electro genetherapy and is still awaiting for its first clinical application.

Increased membrane permeability due to exposure of cells to the electric field (electropermeabilization) is believed to be a consequence of cell membrane electroporation. When a cell is exposed to the electric field, a position dependent transmembrane voltage is induced due to geometric and material properties of the cell and its plasma membrane. If the induced transmembrane voltage is higher than a given threshold value, the membrane area exposed to an overcritical value becomes more permeable. This area of cell membrane represents the area through which molecular transport occurs. The predominant mechanism for molecular transport is difussion due to concentration gradient for small molecules, whereas for larger molecules the transport seems to be more complex and not yet fully understood.

As the induced transmembrane voltage has to exceed certain (reversible) threshold in order to allow for molecular transport, local electric field has to exceed a given electric field threshold. This local electric field depends on electrode geometry, relative electrode geometry/tissue anatomy and passive electric tissue properties. The relevant local electric field can be estimated by means of numerical models (e.g. finite element models) which allow for calculation of electric field in tissue(s) of interest while taking into account the anatomy, tissue dielectric properties and electrode geometry and positioning. Recently models which take into account increase in tissue conductivity due to cell membrane electroporation have been proposed. Exact funtional dependency of tissue conductivity on electric field however still needs to be established and the relation between tissue conductivity changes and membrane electropermeabilization is not clear.

It is important to stress that if the induced transmembrane voltage is higher than given irreversible threshold (also higher than reversible threshold), the cell membrane does not recover and the cell dyes. In electrochemotherapy and even more in electro genetransfection this has to be avoided as much as possible. Again numerical models allow us to plan for positioning of electrodes and determining of voltage to be applied. In principle, by using numerical models a treatment of target tissue can be planned in advance. Since all types of cells were demonstrated to be permeabilizable and electro transfectable, all tissues and cells within the body can in principle be treated by properly designed and positioned electrodes.

# *In vivo* DNA electrotransfer to muscle and tumours: applications in oncology

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DNA electrotransfer is an efficient non-viral approach for the delivery of genes to various tissues *in vivo*. The mechanisms DNA electrotransfer are the electroporation (electropermeabilisation) of the cells of the tissue volume exposed to the electric pulses, and the electrophoretic displacement of the DNA towards the electroporated cells. Therefore combinations of electroporating and of electrophoretic pulses seem quite promising for a safe and very efficient DNA electrotransfert. Such combinations of pulses can be achieved using new equipment recently developed within the frame of the Cliniporator European project.

However, up to the present, most of the experiments concerned by the electrotransfer of therapeutic genes have been performed using trains of identical electric pulses. Several examples of applications of gene transfer in Oncology using such trains of identical pulses will be presented. The delivered genes coded for cytokines or for antiangiogenic factors, which are factors that can act either locally or at distant locations. Therefore genes were electrotransferred either directly to the tumors or to the skeletal muscle where the transfection levels and production of secreted factors can reach high therapeutic levels.

These experiments showed promising results potentially applicable in humans. However, even though electroporating pulses are already delivered to humans in the frame of tumor treatment by electrochemotherapy, the possibility to use DNA electrotransfer in humans must still be confirmed.

# EPR oximetry *in vivo* in cancer therapy

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The oxygen concentration in tumors is considered to be one of the most important environmental factors that affect the response of tumors to different treatments. Therefore we anticipate that the information about the variation of oxygen concentration in tumors could be important for individualizing the timing of different treatments, in order to achieve the optimal response of the organism to the therapy. There is thus a need to follow quantitatively the oxygen concentration in tumors before, during and after different therapies in *in vivo* conditions, so that their use can be translated into the clinical setting. One of the methods that enable measurements of partial pressure of oxygen ($pO_2$) in tissues *in vivo* is EPR oximetry. This is a new and non-invasive method, which is based on the fact that the molecular oxygen is paramagnetic and therefore it broadens the line-width of the other paramagnetic centers in tissues due to the Heisenberg exchange interaction. In this work the basic principles of the method will be described and some examples of oxygen concentration measurements in tumors before and in the process of different treatments will be presented.

For EPR oximetry it is necessary to introduce a paramagnetic probe into the tissue. Usually these are some carbon-based materials such as different coals or chars of carbohydrates, which are usually in the form of fine powder, or lithium phthalocyanine, which is in the form of small crystals. In our examples the paramagnetic probe was implanted into the tumor (center or periphery) and in some cases in the selected normal tissue (skeletal muscle or subcutis) one day before the measurement. EPR spectra line-width, which is proportional to oxygen concentration was measured with time of tumor development or with time after intraperitoneal injection of the tubulin-binding chemotherapeutic drug, vinblastine (VLB) (2.5 mg/kg) or after electrochemotherapy. A decrease in partial oxygen pressure $pO_2$ in tumor was observed during the tumor growth. Tumor oxygenation was also reduced to 50% of pretreatment value 1 h after the treatment with VLB and returned to pretreatment levels within 24 h after the treatment. Reduction in oxygenation of normal tissues, muscle, and subcutis also occurred but was smaller and returned to pretreatment values faster as in the tumors. Similarly, after electro-chemotherapy a reduction in $pO_2$ was observed 2 h after the treatment, with steady recovery to the pretreatment level within 48 h. The overall reduced tumor oxygenation correlated well with the reduced tumor perfusion measured by Patent Blue staining.

These examples demonstrate that EPR oximetry is a sensitive method for monitoring changes in tissue oxygenation before and after different treatments, which may have implications in controlling side effects of therapy and in the planning of combined treatments with other chemotherapeutic drugs or with radiotherapy.

# Electrochemotherapy - effective treatment of tumours in veterinary medicine

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Electrochemotherapy is an antitumour therapy that utilizes locally delivered short intense direct current electric pulses to the tumour nodule and chemotherapy. In Slovenia, in 1999 electrochemotherapy with cisplatin or bleomycin was introduced into veterinary medicine, since there is a need for inexpensive and effective treatment of cutaneous and subcutaneous tumours of various histological types in dogs, cats and horses. Up till now, 19 dogs, 3 cats and 6 horses were treated with electrochemotherapy.

The typical electrochemotherapy procedure consists of intratumoural injection of the drug, followed immediately by application of 8 square wave high voltage electric pulses to the tumour nodule. Electric pulses are delivered to the tumour nodule through either plate electrodes that are placed apposed to the tumour margins or needle electrodes that are inserted into the tumour tissue. In most of the cases, electrochemotherapy with intratumoural injection of cisplatin was used.

In dogs, mammary adenocarcinoma, mast cell tumours, hemangioma, perianal tumours and neurofibroma were treated so far. Altogether, 36 nodules were treated with electrochemotherapy with intratumourally injected cisplatin. In perianal tumour, in some cases electrochemotherapy with cisplatin showed weak response, so electrochemotherapy with intratumourally injected bleomycin was performed as a second line of treatment. Four weeks after the treatment the objective response was obtained in 96% of tumours. Electrochemotherapy with cisplatin was less effective in treatment of mammary adenocarcinoma and fibrosarcoma in cats; objective response of tumours was obtained in 50% (7/14 tumours). Electrochemotherapy with cisplatin was highly effective treatment of sarcoids of different sizes in horses. Objective responses were obtained in 94% (16/17 tumours).

In conclusion, electrochemotherapy with cisplatin or bleomycin is an effective and safe local treatment of different histological types of cutaneous and subcutaneous tumours in cats, dogs and horses. The advantages of this therapy are its simplicity, short duration of treatment sessions, low chemotherapeutic doses, and insignificant side effects, as well as the fact that that the patient does not have to stay in hospital.

# Cell penetrating peptides as drug delivery vectors

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Cell penetrating peptides (CPPs) are a new class of amphipathic peptides, consisting of less than 30 amino acids, bearing the ability of a "spontaneous" cellular internalization that makes them potential drug delivery vehicles. No well established classification scheme for CPPs has been developed yet, however, the most prominent representatives of CPPs are penetratin, tat, MAP, transportan, polyarginines, and pVEC. Penetratin (pAntp) is a 16-amino acid long peptide from the third helix of the *Drosophila* Antennapedia homeodomain (amino acids 43-58), which has a sequence-specific DNA binding property. Tat is an 86-amino acid protein of human immunodeficiency virus 1 (HIV-1) which is able to translocate through the plasma membrane. Region 37-72 was shown to be sufficient for the translocation. Model amphipathic peptides (MAPs) describe a group of peptides derived from the 18-amino acid long α-helical amphipathic synthetic peptide with regular sequence of alternative hydrophilic and hydrophobic amino acids. Transportan is a 27 amino acid-long chimeric peptide, with 12 amino acids from the neuropeptide galanin in the amino terminus, 14 amino acids from mastoparan in the carboxyl terminus, and one lysine in-between. Polyarginines are short oligomers of arginine that entered cells to much greater extent than corresponding short oligomers of histidine, lysine, and ornithine. pVEC, an 18-amino acid-long peptide with amphipathic character, was derived from murine vascular endothelial cadherin. It is the first CPP generated from a membrane-spanning glycoprotein. CPPs penetrate into various cells and tissues, as established both *in vitro* and *in vivo*. CPPs are able to translocate cargoes across the cell membrane. Oligonucleotides, proteins, peptide nucleic acids (PNA), liposomes, plasmids, nanoparticles, adenoviruses and different small hydrophilic molecules have been successfully transported over plasma membrane so far; for some cargoes, their biological response has been detected after internalization. Cargo is usually attached to CPP via covalent bond (-S-S-, amide bond, etc), but can be internalized also when tightly non-covalently bound to the carrier peptide. Penetration mechanism of CPP internalisation has not been disclosed yet. It seems to be mainly receptor- or/and energy-independent but for some CPPs, the uptake is mediated at least partially by endocytosis. CPPs are promising vectors for the non-viral delivery of DNA, PNA, and RNA (siRNA) fragments, suitable for gene-therapy. For cancer therapy the tissue selective CPPs would be of great interest. The clue for the improved selectivity is the knowledge of internalization mechanism. Additional problem in practical use of CPPs are their potential side effects. Some of CPPs disrupt the cell membrane at high concentration and make pores in it, while some bind to G-proteins and interfere with signal transduction processes. Therefore, much effort has been recently invested to clarify penetration mechanism and to design less toxic CPPs.

# Colloidal drug delivery systems in cancer therapy

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The targeting of cancer tissue is an area of extensive investigation, with the aim of delivering therapeutic moieties with high specificity. Cancerous cells and tissues can be characterized by their unique biochemical and physiological properties, some of which are exploited in developing therapies. A complication with cancer therapy is the fact that anticancer agents invariably attack healthy cells as well as tumour cells. This is due to the fact that anticancer drugs generally act by interfering with the replication cycle of cells.

One strategy could be to associate anticancer drugs with colloidal drug delivery systems (CDDS), in order to overcome non-cellular and cellular based mechanisms of resistance and to increase selectivity of drugs towards cancer cells, while reducing their toxicity towards normal tissues. CDDS may be defined as being submicronic ( $< 1$ μm) particles. According to the materials and processes used for preparing the CDDS, micelles, liposomes, and nanoparticles can be obtained. If designed appropriately, CDDS may act as drug carriers able to target tumor tissues or cells with a certain specifity, while protecting the drug from premature inactivation during its transport. An attractive feature of CDDS is their ability to carry a large payload of drug. The drug may be encapsulated within the particle architecture, or by adhesive or electrostatic binding to the particle surface, or by conjugation to the particle surface terminal groups.

The presentation will first review CDDS and how, loaded with anticancer drug, they can increase the drug concentration in cancer tissue, enhancing antitumour efficacy. There are several examples of both passive and active drug targeting, because neoplastic tissues comprise three subcompartments (vascular, interstitial and cellular) with specific properties.

Abnormalities of tumour blood vessels, compared with vessels in normal tissue, are an important characteristic which has been employed as a strategy in passive drug targeting: the pore cutoff size ranges between 10 and 500 nm, enabling CDDS to be localised within tumours to a greater extent than in the healthy tissue. The tumour interstitial compartment is essentially a hydrophilic gel, where the transport of an anticancer drug will be governed by its physiological properties (i.e. pressure, composition, structure, charge) and by physicochemical properties of the drug molecule and carrier.

The most important feature is that the CDDS may also have an influence at the cellular level. Tumour cells are recognised by the body as foreign, displaying antigens, that distinguish them from healthy cells. This difference has been employed in active targeting for enhancing the selectivity of drugs towards malignant cells. Surface engineering of CDDS (antibody-mediated delivery) may be used to modify their biological profile and activity. Particles can be endocytosed or phagocytosed by cells with resulting cell internalization of the loaded drug. The contribution of CDDS in cancer chemotherapy and gene therapy will certainly grow, providing that more efficient strategies for tumour targeting and protection of drug activity are developed.

# Some new applications of electroporation in biomedicine

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Knowledge about mechanisms involved in antitumour effectiveness of electrochemotherapy, a combination of chemotherapy with subsequent local delivery of electric pulses to the tumour, opened new possibilities for biomedical application of electric pulses and electrochemotherapy in cancer treatment. Lately, at the Institute of Oncology Ljubljana, some of these new applications have been evaluated, such as tumor radiosensitisation by increased radiosensitizing drug delivery, increased efficacy of some therapeutic approaches such as bioreductive drugs and hyperthermia, as well as electrogenetherapy.

Some chemotherapeutic drugs interact with radiation therapy. Among the radiosensitizing drugs are also bleomycin and cisplatin. Combined modality therapy with cisplatin or bleomycin and radiation was improved by electroporation of tumors. Irradiation dose needed to cure 50% ($TCD_{50}$)of the treated LPB fibrosarcoma tumors was lowered by 1.9-fold when electrochemotherapy with bleomycin was combined with irradiation. Radiosensitisation of tumours with electrochemotherapy with cisplatin was less effective and resulted in 1.6-fold lower $TCD_{50}$ compared to the irradiated tumours only.

Application of electric pulses was shown to modulate tumour blood flow. Reduced tumour blood flow and partial oxygen pressure ($pO_2$) are both consequences of applied electric pulses. The reduction of $pO_2$ could selectively activate bioreductive drugs that exhibit better cytotoxic effect on hypoxic cells than on the cells that are well oxygenated. In a study combining application of electric pulses with bioreductive drug tirapazamine we showed that *in vivo* treatment of subcutaneous tumours with combination of tirapazamine and application of electric pulses significantly enhanced tumour response compared to single treatments.

In addition, tumour hypoxia induced by application of electric pulses can provide improved therapeutic conditions for the use of hyperthermia, since tumour cells are more sensitive to heat in sub-optimal physiological conditions. When hyperthermia was performed immediately after application of electric pulses, when tumour perfusion was maximally reduced, greater than additive antitumour effectiveness was observed with 43% complete responses of the tumours

Application of electric pulses could be also used for introduction of plasmid DNA into cells and tumours *in vivo*. Electric pulses used for DNA transfer differs from those that are used for electrochemotherapy and optimisation of pulse parameters with respect to tumour type should be done in order to obtain good transfection efficiency. In a study on four different tumour models, we found that electroporation-assisted gene delivery with plasmid DNA, employing long electric pulses with low amplitude, yielded significantly better reporter gene expression than short electric pulses with high amplitude. In addition, electroporation assisted delivery of therapeutic gene p53 into solid tumours in mice showed the feasibility of this approach for cancer gene therapy.

# Electrochemotherapy with cisplatin given intratumourally in treatment of malignant melanoma patients

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Electrochemotherapy combines administration of nonpermeant or poorly permeant chemotherapeutic drugs with application of electric pulses to the tumours in order to facilitate the drug delivery into the cells. Thus, enhanced drug delivery can substantially potentiate chemotherapeutic drug effectiveness, locally at the site of the cell electroporation without affecting drug effectiveness in the tissues that were not exposed to electric pulses. Based on numerous preclinical studies on electrochemotherapy using either bleomycin or cisplatin, the first clinical study on electrochemotherapy with bleomycin was performed in 1991, demonstrating good antitumor effectiveness on cutaneous metastases of head and neck carcinoma patients. After that initial study, several clinical studies on electrochemotherapy using bleomycin or cisplatin administered locally or systemically were performed. Cutaneous metastases of different tumours were treated, such as head and neck squamous cell carcinoma, malignant melanoma, basal cell carcinoma, adenocarcinoma of the breast and salivary gland, hypernephroma, Kaposi sarcoma and transitional cell carcinoma of the bladder. All together 418 nodules in 85 cancer patients were treated. Overall results of these studies show that electrochemotherapy is effective treatment; objective responses were obtained in 48 % to 100 % of the treated nodules.

The aim of the present study on electrochemotherapy with cisplatin injected intratumourally was to treat malignant melanoma patients with progressive disease in order to alleviate side effects to the patients, to re-evaluate the effectiveness of electrochemotherapy on a similar group of patients as in the previous study (2) and to gain experience in order to further optimise treatment procedure.

In the study, 14 patients with progressive disease of malignant melanoma were included. Tumours nodules (211 nodules) that were treated were of varying sizes (from 4mm to 3 cm in diameter). The treatment was performed by intratumoural injection of cisplatin (~ 1mg/1cm$^3$), which was followed by application of electric pulses, delivered using plate electrodes with inner distance of 7 mm. Eight electric pulses of 1300 V/cm (amplitude/distance ratio), 100 μsec long, with frequency 1 Hz, which were applied to the nodule, were generated by electroporator Jouan GHT 1287. The nodules that were bigger than the distance between the electrodes were treated by consecutive applications of electric pulses until the whole tumour area was covered. Patients were regularly checked for response in 2-4 weeks interval. Four weeks after the treatment, in most cases, partial or complete responses of the treated nodules were obtained (82%). In some cases, when the tumour nodule regrew between the two follow-ups, or when the first session did not result in complete regression of the nodule, additional electrochemotherapy sessions were needed.

In conclusion, the results of the present study on malignant melanoma patients demonstrate that the described protocol for electrochemotherapy is effective and reproducible resulting in high percentage of partial and complete responses.

# Potentiation of radiation response of tumors by electrochemotherapy

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**Introduction:** Electrochemotherapy is an antitumor treatment that utilizes short high voltage electric pulses to increase the effectiveness of chemotherapeutic drugs in cells and tumors. Electric pulses (electroporation) permeabilize tumor cells to allow poorly permeant drugs such as bleomycin and cisplatin to enter the cells. Bleomycin and cisplatin have besides cytotoxic also radiosensitizing effect. When bleomycin is present in the cell, it causes single- and double-stranded breaks in DNA by production of a free radical complex utilizing oxygen and ferrous ion. Cisplatin exert its cytotoxic effect by forming intrastrand or interstrand crosslinks in DNA molecule and also by crosslinking DNA molecule with chromosomal proteins. Our previous study showed transient reduction of tumor blood flow and therefore lower oxygen level in tumors after the application of electric pulses.

**Aim:** In this study a physical drug delivery method, electroporation, was used to facilitate bleomycin and cisplatin delivery into the cells and tumors with the aim of increasing radiation response. In addition, because it is known that radiation response of tumors is approximately 3-times higher in the presence of oxygen, the effect of electroporation on radioresponse of tumors was also evaluated in our study.

**Materials** and methods: LPB sarcoma cells and tumors were treated either with bleomycin, cisplatin, electroporation or ionizing radiation, and combination of these treatments. *In vitro*, radiation response was determined by colony forming assay, while *in vivo*, treatment effectiveness was determined by local tumor control ($TCD_{50}$). Time dependence of partial oxygen pressure in LPB tumors after application of electric pulses was measured by electron paramagnetic oxymetry.

**Results:** The radiosensitizing effect of bleomycin and cisplatin on LPB tumor cells was increased by electroporation. This combined treatment was more effective when using bleomycin, compared to cisplatin. Electrochemotherapy with bleomycin potentiated cytotoxicity of irradiation by 1.53-fold and by 1.35 in the case of electrochemotherapy with cisplatin. Increased radiosensibilization of LPB tumors with bleomycin and cisplatin after application of electric pulses was determined also *in vivo*. Irradiation dose needed to cure 50% ($TCD_{50}$) of the treated tumors was lowered by 1.9-fold when electrochemotherapy with bleomycin was used. Radiosenzibilization of tumors with electrochemotherapy with cisplatin was less effective, resulted in 1.6-fold lower $TCD_{50}$ compared to the irradiated tumors only. Application of electric pulses to the tumors induced profound but transient reduction of tumor oxygenation. Although tumor oxygenation partially restored at the time of irradiation, partial oxygen pressure especially in the tumor center was still low enough to affect radiation response of tumors alone or in combination with electrochemotherapy. Conclusion: Our study showed that radiosensitizing effect of bleomycin and cisplatin was increased due to their uptake into the LPB tumor cells and tumors after the electroporation.

# New avenues for anti-proteolytic metastasis therapy: superiority of selectivity-optimized synthetic matrix metalloproteinase and serine proteinase inhibitors

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Since matrix metalloproteinases (MMPs) or serine proteases like uPA facilitate tumor cell invasion and metastasis, a great effort has been devoted to develop protease inhibitors as cancer therapeutic agents. A loss of momentum was experienced in the field when most clinical trials with first generation broad-spectrum MMP-inhibitors were discontinued without proving a reduction in mortality or extending the disease-free interval. Unless we undertake a profound change in our strategy to design protease inhibitors, we will not be able to prevent future failures and entirely loose one promising route in metastasis-inhibition. We were the first to demonstrate a liver metastasis-promoting effect of a broad spectrum MMP inhibitor of human ovarian, oesophagus, and breast carcinomas as well as a murine T-cell lymphoma in mouse metastasis models. We then identified MMP-9 as one of the important metastasis-associated proteins and showed, with selectivity-optimized second generation MMP inhibitors, a close correlation between MMP-9 selectivity and anti-metastatic efficacy. Next, we screened a series of closely related structurally fine-modulated serine protease-inhibitors for their anti-metastatic efficacy in a T-cell lymphoma liver metastasis assay in mice. Using these inhibitors with distinct selectivity-profiles for prototypical serine proteases (uPA, plasmin, thrombin, factor Xa, trypsin), allowed selectivity/anti-metastatic efficacy correlations and revealed factor Xa or a factor Xa-like protease to be another important metastasis-associated gene in this model. Furthermore, we found that among the protease inhibitors employed that have affinities in the nanomolar range, the strategy of selectivity-optimization is superior to further improvement of affinity to significantly enhance anti-metastatic efficacy. This appreciation may be important for the future rational design of new anti-proteolytic agents for cancer therapy.

# Modifying TNF alpha to improve its potential for cancer therapy

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Tumour necrosis factor alpha (TNF) has reached high attention in research and therapy for two main reasons. The currently most popular one is the role of TNF in various pathologies, e.g., rheumatoid arthritis and Crohn disease. Blocking the excess of TNF is the main and relatively successful approach in these cases. The other reason that TNF persists in the research focus is its ability to kill tumour cells *in vitro*, and even more important, its curative potential for certain cancers, e.g., inoperable sarcoma and melanoma. Both pathological and curative aspects of TNF must take into account its systemic toxicity. Therefore, the first part of the talk will provide short overview on modifying TNF performed to reduce its systemic toxicity or to increase anti-tumour activity by more specific targeting or more selective action on tumour cells.

In the second part, our recent experiments with various TNF analogues *in vitro* will be presented. We have been exploring the effect of charge modification on the surface of TNF trimer, preferentially in the tip region, which is oriented towards the cell during the interaction with TNF receptors. Introduction of positive charge leads to increased specific cytotoxicity on certain tumour and transformed cells, e.g., mouse cells L929. It is interesting that variation of isoelectric point of TNF analogues, due to mutations in the tip region, shows similar effects on cytotoxicity on selected mouse (L929 and WEHI-164) as well as human (KYM-1D4) cell lines. The important message from this experiment might be that the interaction mechanisms on mouse and human cells are similar enough allowing the use of mice as relevant model for preclinical studies. In another set of experiments the charge on the cell surface was also modified, e.g., by exposing cells to various heparinases. Removal of negatively charged heparan sulfates reduces the specific cytotoxicity of analog LK-805 bearing positive charge in the tip region. This indicates that cells having increased number of negatively charged glycosaminoglycans might have additional attractive interaction with LK-805 or similar analogues leading to local on-the-surface enrichment of TNF molecules. Tumor cells, microvascular endothelial cells as well as hepatocytes are among the most important target cells to be checked with newly designed "charged" TNF analogues. Similar interaction behavior might also be expected with many other cytokines, either native or modified *in vitro* for therapy.

# Cathepsin B in tumour progression and possible applicability of cathepsin B neutralising 2A2 monoclonal antibody in cancer treatment

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Lysosomal cysteine protease cathepsin B plays a central role in degradation of extracellular matrix proteins, facilitating tumour cell invasion and metastasis. Cathepsin B activity can be inhibited by specific inhibitors, however, the inhibitors known so far are not specific *in vivo*. Improved specificity can be achieved by 2A2 monoclonal antibody, binding to the EPGYSP epitope at the exposed part of cathepsin B molecule in the occluding loop and neutralising its enzymatic activity. We showed that 2A2 monoclonal antibody is more effective inhibitor of cathepsin B in *in vitro* invasion assays than specific synthetic inhibitors, presumably as a result of antibody internalisation in living cells and inhibition of extracellular and intracellular cathepsin B. Importance of cysteine proteases, including cathepsin B, for tumour cell invasion and metastasis was confirmed also *in vivo*, on lung metastasis model in mice, using B16-F10 mouse melanoma cells. Chicken cystatin decreased the number of metastases in both BALB/c mice and in heterozygotes of stefin B knock out mice. Although 2A2 antibody predominantly binds human cathepsin B, and to a lower extent murine cathepsin B, its inhibitory effect was also demonstrated in lung metastasis assay. Our results show, that inhibitors acting on intracellular and extracellular cathepsin B are more effective in preventing tumour cell invasion, and, furthermore, demonstrate the applicability of 2A2 monoclonal antibody in cancer treatment.

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P10. Vida Stegel: Detection of telomerase RNA in the plasma of patients with breast carcinoma.

P11. Nick J. Osborne: *In vitro* investigations on the variation in induction of apoptosis in gliomas by antidepressants

P12. Alenka Grošel: Electrogenetherapy with p53wt combined with cisplatin: an *in vitro* study on human tumour cell lines with different p53 status

P13. Mekdes Debela: Cloning, expression, purification, refolding, and initial biochemical characterization of human tissue kallikreins

P14. Mateja Cegnar: Passive targeting of cysteine protease inhibitor cystatin into tumour cells by using poly(lactide-co-glycolide) nanoparticles

P15. Tatiana A. Korolenko: Cystatins and cysteine proteinases in murine and human tumours

P16. Tomaž Langerholc: Unusual localization and inhibitory properties of cystatin F in U937 cells

P17. Tatiana A. Khalikova: Cathepsins B, L and D activity and cystatin C concentration in mice with sensitive and resistant to cyclophosphamide variants of lymphosarcoma LS

P18. Mads Nordahl Svendsen: Influence of open versus laparoscopically assisted colectomy on plasma VEGF and VEGFR1

P19. Miha Trinkaus: The role of cathepsin B in invasion and apoptosis of glioblastoma U87 cells

P20. Irena Zajc: Regulation of cathepsin L affects the invasiveness and apoptosis of glioblastoma U87 cells

P21. Bojana Žegura: The role of reduced glutathione in microcystin-LR induced genotoxicity

P22. Uroš Rajčevič: Proteomic approach to identification of differentially expressed proteins in two types of stomach cancer

P23. Adaleta Mulaomerović: Cathepsins X, S and cystatin C in sera of patients with B-cell lymphoma

P24. Mateja Gabrijel: Quantification of cell hybridoma yields with confocal microscopy and flow cytometry

P25. Ira Kokovič: SYT-SSX fusion genes and prognosis in synovial sarcoma: a retrospective study of 37 slovenian patients

P26. Maja Čemažar: Schedule-dependency between vinblastine and adriamycin in EAT tumours in mice

P27. Janja Plazar: Do reactive oxygen species play role in 2-amino-3-methyl-imidazo [4,5-f]quinoline (IQ) induced genotoxic effects?

# ABSTRACTS OF POSTERS

# Aberrant expression, processing and degradation of dystroglycan in squamous cell carcinomas

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The α- and ß-dystroglycan (DG) proteins are involved in epithelial cell development, formation of the basement membrane and maintenance of tissue integrity. Recently, specific changes have been described in dystroglycans expression in some cancers.

We analysed the expression and localisation of α-DG and ß-DG using Western blotting, immunohistochemistry and RT-PCR in samples of normal oral mucosa, oral squamous cell carcinoma and cancer cell lines. The α-DG and ß-DG were localised in the basal layers of normal oral mucosa. However, ß-DG expression in cancer showed evidence of aberrant expression, processing and degradation and the α-DG was altered in all oral cancer samples and cell lines, despite persistent presence of dytroglycan mRNA in cancer cells. Using matrix metalloproteinase inhibitors, we determined that the ß-DG degradation in carcinoma cell lines can be mediated but did not exclusively dependent on a function of a particular MMP. We currently extend our analyses into other cancer types. Considering the multifaceted role of dystroglycan in epithelial development, it appears that the role of dystroglycan degradation in cancer growth and spread, although currently not understood, may be significant.

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# Development of a canine-specific radioimmunoassay for cathepsin B and its use for the evaluation of cathepsin B expression in canine mammary tumour cell line U335 (CMT-U335)

*Alenka Nemec,*[1] *Marinka Drobnič-Košorok,*[2] *Jeannette Wolfswinkel,*[3] *Elpetra Timmermans Sprang,*[3] *Jan Mol*[3]

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The lysosomal cysteine proteinase cathepsin B (CB) has been implicated in the progression of a variety of human tumours. Although human CB is one of the most extensively investigated very little is known about canine cathepsin B and its role in canine mammary tumours, which are the most frequent neoplasms affecting the bitches. The incidence is three times as high as in woman. The pathogenesis of canine mammary tumours is not fully understood, but it is generally assumed that hormones and growth factors play a role in tumourigenesis.

The aim of the study was to develop a sensitive radioimmunoassay for the evaluation of the expression of CB in canine mammary tumour cell line U335 (CMT-U335) after induction of apoptosis and treatment with differentiating agents. Therefore, canine CB was purified from liver cytosol and next used for immunization of rabbits. Using radioiodinated CB and the obtained rabbit antiserum CB concentrations could be measured in culture medium and lysates of CMT-U335.

It is concluded that a reliable method has been developed to measure canine CB concentrations *in vitro* which enables us to investigate the epithelial CB production in more detail in the future. The results of *in vitro* study in CMT-U335, which included 24 hours stimulus showed that cytosolic concentrations of CB are influenced by agents, which are able to affect cell growth, differentiation or are able to induce apoptosis. Staurosporine, C2 ceramide (C2C) and TNF-α were able to induce apoptosis. Morphological features of apoptosis were accompanied with statistically significant decrease in CB concentrations. Although insulin like growth factor-I is known to protect against apoptosis induced by a wide range of agents in a variety of cell lines it could not rescue CMT-U335 cells from C2C induced apoptosis. Retinoic acid and synthetic progestin ORG 2058 were able to increase CB concentrations statistically significant, which might indicate their potential role in canine mammary tumourigenesis. Progesterone and medroxy progesterone acetate did not affect CB concentrations, while cortisol decreased CB moderately. From this it is concluded that a limited progestin-stimulated increase of cytosolic CB concentrations may play a role in canine mammary tumourigenesis that can be inhibited by glucocorticoids.

The results of *in vitro* study in CMT-U335 in some cases oppose to the data from the literature, where different human cell lines were used. We assume that the results obtained might be attributed to the specific characteristics of the cell line used and therefore to the cell line specific response to different stimuli.

# Induction of cytotoxic T cell responses *in vitro* using monocyte derived dendritic cells pulsed with tumor lysates or transfected with total tumor cell RNA

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Because of their potent T cell stimulatory capacities, dendritic cells (DC) have been widely used to trigger specific cytotoxic T cell (CTL) responses against various tumor-associated antigens (TAA). Recent research indicates that the loading of DC with the entire TAA spectrum might be far more efficient in evoking anti-tumor immune responses, when compared to the use of a single TAA.

Therefore we decided to study in vitro induction of CTL responses against unidentified hepato-biliar cancer TAA using monocyte derived DC. Monocytes were isolated from buffy coats of healty individuals and subsequently differentiated into immature DC that were pulsed either with tumor lysate or total tumor cell RNA, isolated and prepared from surgically obtained biopsy material. The total tumor cell RNA was also transfected into DC by using cationic lipid DOTAP. After maturation, the TAA-loaded DC were used as stimulators of autologous lymphocytes. The induction of primary CTL responses was measured in a standard $^{51}$Cr cytotoxicity assay.

We showed that CTLs, primed with TAA presenting DC were capable of specifically recognizing and lysing the same TAA expressing targets *in vitro*. The pulsing of DC with tumor lysate as well as their lipofection with total tumor cell RNA were more effective in inducing *in vitro* CTL responses than pulsing of DC with total tumor cell RNA. Therefore, the first two approaches seem to be promising methods for the preparation of DC based vaccines that could be used for the treatment of solid tumors. Since amplified RNA can be prepared from only few tumor cells, the total tumor RNA-transfected DC could also be used for immunotherapy of cancer patients with a minimal tumor burden, where the preparation of sufficient amounts of tumor lysates would be impossible.

# New sacharide derivatives of indolo[2,3-B]quinoline as cytotoxic compounds and topoisomerase II inhibitors

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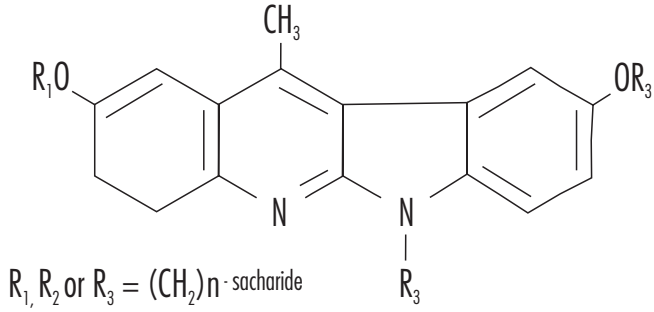
Some of alkyl- and alkylamino- derivatives of 6H-Indolo[2,3-b]quinolines are known to be active antiproliferative and cell cycle modulating compounds. Their cytotoxic properties are at least in part due to DNA intercalation ability and topoisomerase II inhibition activity[1,2].

To improve physicochemical and biological properties of 6H-indolo[2,3-b]quinolines the series of new, glycoside (C-2, C-9 and N-6) derivatives (Scheme) was designed and synthesized:




The influence of different carbohydrate units (D-glucose, D-lactose, L-rhamnose, L-acosamine, L-daunosamine), position of attachment and linker size on cytotoxic properties and topoisomerase II inhibition activity were tested.

There is an obvious relationship between structure and activity. Not only cytotoxicity could be enhanced by substituent changes but also a significant correlation between topoisomerase II inhibition and cytotoxic properties is observed.

1- F. Pognan, J.M. Saucier, C. Paoletti, L. Kaczmarek, P. Nantka-Namirski, M. Mordarski and W. Peczynska-Czoch, Biochem. Pharm., 44, 2149-2155 (1992).

2- W. Peczynska-Czoch, F. Pognan, L. Kaczmarek, J. Boratynski, J. Med. Chem., 37, 3503-3510 (1994).

# Diagnosis and classification of spontaneously developed and radiation-induced murine haematopoietic neoplasms

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The Hematopathology Subcommittee of Mouse Models of Human Cancer Consortium (MMHC) proposed a classification that can be readily compared with the human WHO classification but that also appropriately delineates the diseases as they occur in mice. The mouse lymphoid and nonlymphoid neoplasms develop spontaneously in certain strains, genetically engineered mice (GEM) or follow induction with ionising radiation, chemical carcinogens or viruses.

The aim of this study was to classify:
- spontaneously developed haematopoietic neoplasms in recombinant congenic strain OcB/Dem
- spontaneously developed haematopoietic neoplasms in AKR/W mice carrying endogenous ecotropic provirus inducing potential lymphomas
- radiation-induced haematopoietic neoplasms in Bc recombinant congenic strains (CcS17 x CcS2) x CcS2.

Previously we classified those neoplasms according to Pattengale and Taylor (1983) - the classification parallel to the Lukes-Collins (1974) proposals for human haematopoietic neoplasms. Recently we have reclassified them following the WHO classification of murine lymphoid and nonlymphoid neoplasms (also known as "Bethesda proposals for classification of lymphoid neoplasms in mice" and "Bethesda proposals for classification of nonlymphoid haematopoietic neoplasms in mice" 2002) Criteria for the diagnosis and the classification will be presented.

Morphology of mouse lymphomas and nonlymphoid haematopoietic neoplasms was assessed by microscopic examination of paraffin-embedded tissues. Estimating the phenotype of lymphomas and nonlymphoid neoplasms was based on histochemical, immunohistochemical reactions (IH) and flow cytometry (FCM). ASD and AS-BI histochemical reactions were performed on air-dried imprints. Two immunohistochemical methods were used: ABComplex and MOM® (Mouse on Mouse) on paraffin sections and air-dried imprints with 5 monoclonal antibodies (MAbs). Flow cytometry analysis of neoplastic single-cell suspension was based on 10 MAbs.

# A highly selective mechanism-based gelatinase inhibitor: reduction of metastasis and increase of survival in a highly aggressive T-cell lymphoma model

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Targeting metastasis in therapeutic regimens is still a major challenge in the treatment of cancer patients. Evidence has shown that the matrix metalloproteinases (MMPs) family of zinc endopeptidases plays a key role in promotion of tumor growth and metastatsis. In particular, gelatinases (MMP-2 and MMP-9) have been shown to be directly involved in metastasis. Therefore, gelatinase inhibition has become an important target for new anti-metastatic therapies. We have previously reported an innovative approach to MMP inhibition involving irreversible mechanism-based MMP inhibitors. Our approach has been a point of departure from current concepts in the MMP inhibition arena and, as a proof of concept, we have developed a prototype inhibitor, designated SB-3CT, that is highly specific for the gelatinases (J. Am. Chem. Soc., 122, 6799-6800, 2000). Previously, we identified MMP-9 (gelatinase B) as one of the important metastasis-associated proteases in a murine T-cell lymphoma liver metastasis model (Cancer Res., 62, 5543-5550, 2002). In the present study, we report the efficacy of SB-3CT administration in inhibiting liver metastases in the murine T-cell lymphoma model. Treatment of mice with a regimen of 5 to 50 mg/kg/day of SB-3CT caused a 30 % to 75 % reduction of metastasis, respectively, when compared to vehicle-treated mice. In addition, treatment with 50 mg/kg/day of SB-3CT significantly inhibited the growth of metastases. Furthermore, survival was significantly increased. When compared to other synthetic MMP inhibitors tested in this model (Cancer Res., 62, 5543-5550, 2002), SB-3CT exhibits a significantly higher inhibitory effect. These studies demonstrate the importance of selective inhibition of MMPs in the treatment of highly aggressive tumors and provide new hopes for the implementation of new clinical trials with more effective inhibitors.

# Cytotoxicity of different platinum(II) complexes on different human tumor cell lines

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Cisplatin, oxaliplatin and carboplatin are platinum complexes that are already used in the clinical practice. Because of their toxicity against normal cells and resistance of tumor cells, new platinum(II) complexes were synthesized and tested for their antitumor efficiency.

The aim of our study was to determine *in vitro* cytotoxic activity of new platinum(II) complexes: [Pt(succ-O,O)(Hmpy)$_2$] **(1)**, [Pt(mal-O,O)(Hmpy)$_2$] **(2)**, [Pt(cbdc-O,O)(Hmpy)$_2$] **(3)**, [Pt(cbdc-O,O)(Ampy)$_2$] **(4)** (succ = succinate; mal = malonate; cbdc = 1,1-cyclobutanedicarboxylate; Hmpy = 3-hidroxymethylpyrimidine; Ampy = 2,2-aminomethylpyrimidine) in comparison with cisplatin (CDDP), carboplatin and oxaliplatin on four different human tumor cell lines (bladder carcinoma T24, ovarian carcinoma IGROV1, resistant ovarian carcinoma IGROV1/RDDP and mammary carcinoma MCF7).

Cells were plated into 96-well microtiter plates at a concentration 3000 or 5000 cells/well in 50 µl medium. After allowing time for cells to attach, 50 µl of different concentrations of the complexes were added to the wells (8 wells/drug concentration). Cells were incubated with platinum complexes 3 or 5 days at 37°C in humidified atmosphere containing 5% $CO_2$. Cytotoxicity of the compounds was determined by the colorimetric MTT assay (Mosmann T *et al*, J Immun Meth, 1983: 65:55-63). The inhibitory concentration of the drug that reduced survival of cell to 50% ($IC_{50}$) was determined for each cell line.

Human bladder carcinoma cell line T24 was sensitive to CDDP, oxaliplatin and complex **1**, but not to the other platinum complexes. On this cell line the most effective was oxaliplatin, the inhibitory concentration $IC_{50}$ was 1.1 µM. The most sensitive cell line to all platinum complexes as determined by $IC_{50}$ value was human ovarian carcinoma IGROV1 cell line. The $IC_{50}$ values for CDDP, which was the most cytotoxic agent for this cell line was 1.1 µM. The least effective was complex **4**, the $IC_{50}$ value was 74.5 µM. IGROV1/RDDP cell line, a CDDP-resistant subclone of IGROV1 cell line, was also less sensitive to other complexes compared to IGROV1 cell line. Human mammary carcinoma cell line MCF7 was sensitive to cisplatin and oxaliplatin, their $IC_{50}$ values were 2.1 µM and 11.6 µM, respectively. This cell line was less sensitive to complexes **1, 2, 3, 4,** and carboplatin.

The results of our study showed that cisplatin and oxaliplatin were the most effective in reducing cell survival on all cell line used. Cytotoxicity of complex **1** was comparable to cytotoxicity of carboplatin, a chemotherapeutic drug that is already used in clinical practice. The rest of the newly synthesized complexes showed minor cytotoxic effect on the used cell lines. Therefore, pronounced cytotoxic effect of complex **1** against all cell lines tested complex **1** warrants further *in vivo* studies.

# Effective treatment of perianal tumours in dogs with electrochemotherapy

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Electrochemotherapy is an antitumour therapy that utilizes locally delivered short intense direct current electric pulse to the tumour nodule and chemotherapy. The aim of the present study was to evaluate the electrochemotherapy treatment of perianal adenomas and adenocarcinomas of different sizes in dogs.

Between March 2000 and July 2003, 12 patients with all together 26 measurable cutaneous were included in the study. Before treatment, the dogs were sedated. Electrochemotherapy consisted of intratumoural administration of cisplatin at doses ranging from 0.5 to 2.5 mg/100 mm³ tumour volume, followed by application of electric pulses to the tumour. If the treatment with cisplatin as the first chemotherapeutic drug was not successful, electrochemotherapy with bleomycin was performed, with bleomycin doses ranging from 2 to 3 mg/100 mm³ tumour volume. Square wave electric pulses of 100 μsec, 910 V amplitude (amplitude to electrode distance ratio 1300 V/cm), frequency 1 Hz were delivered through two parallel stainless steel electrodes (thickness 1mm; width 7mm, length 8mm, with rounded tips and an inner distance between them 7 mm) with an electroporator Jouan GHT 1287. Each run of electric pulses was delivered in two trains of four pulses, with 1s interval, in two perpendicular directions. The interval between cisplatin or bleomycin administration and the application of electric pulses was 1-2 min. The treatment response was evaluated according to WHO classification (complete response (CR); partial response (PR); no change (NC) and progressive disease (PD)).

Responses to treatment were assessed 4 weeks after therapy; in 91.6% of all tumours treated with electrochemotherapy objective responses (OR) were obtained, 8.4% of tumours were in NC and none of the tumours went in the PD. Observation period was between 1 and 42 months. At the end of observation period 87.5% of tumours were in OR, 12.5% in NC and none of them in PD. According to the tumour size, tumours were divided into two subgroups. In the first subgroup (tumours with volume less than 1 cm³) an excellent response to the treatment after 4 weeks was achieved: 100% of tumours were in OR. At the end of observation period even more tumours responded completely (86.8%) and the rest of them were in PR (13.2%). In the second subgroup (tumours with volume bigger than 1 cm³) 77.7% of OR and 24.2% of NC were obtained after 4 weeks. At the end of observation the percentage of CR increased to 22.2%, 55% of tumours were in PR and only 22.2% in NC. To achieve above-mentioned results, average number of electrochemotherapy sessions was 1.6 and in range from 1 to maximum 4 sessions per patient. There were no major local or general side effects noted.

This study shows that electrochemotherapy with cisplatin or bleomycin is an effective treatment of perianal tumours in dogs. The advantages of this therapy are simplicity, short duration of treatment sessions, low chemotherapeutic doses, and insignificant side effects, as well as the fact that it can be performed on outpatient basis.

# Tumour suppresors of MAGUK family in apoptosis

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MAGUKs (membrane associated guanylate kinases) are components of protein complexes that are involved in cell-cell adhesion. With their protein interaction domains they bind other proteins of cell adhesion complexes and link them to the cytoskeletal proteins. They also have a role in tumour formation and progression. For successful transformation HPV must degrade MAGI-1 by marking it for proteosomal degradation. DLG is involved in Wnt/ß-catenin signalling pathway and mutations in dlg gene may result in tumour development.

Apoptosis is a physiological process, important in eliminating superfluous, damaged, infected and thus potentially dangerous cells. One of the important steps in apoptosis is the dismantling of cell-cell contacts, and proteins involved in the formation of these contacts represent potential targets for caspases during apoptosis.

We have discovered that aforementioned members of the MAGUK family, hDLG (SAP-97, DLG1) and MAGI-1, are cleaved by caspases during apoptosis in HaCaT epithelial cell line. After the cleavage, proteins dissociate from membrane to the cytosol and their structural role is probably lost, leading to dismantling of cell-cell contacts in dying cells. We have identified Asp761↓Ser762 as the primary caspase cleavage site in MAGI-1. Mutating Asp761 to Ala prevented MAGI-1 cleavage by caspases. Furthermore, HaCaT cells overexpressing MAGI-1 noncleavable mutant exhibited delayed cells' dissociation during staurosporine-induced apoptosis. We believe that cleavage of proteins from MAGUK family by caspases is thereby an important early step in the disassembly of cell-cell contacts during apoptosis.

# Detection of telomerase RNA in the plasma of patients with breast carcinoma

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**Introduction:** The "conventional" tumor markers (CEA, CA 15-3) are predominantly circulating proteins. Since it was clearly proved that detectible amounts of nucleic acids are present in the human serum, they are considered as potential tumor markers in cancer patients. The PCR based technologies allowed amplification of small amounts of DNA or cDNA, thereby increasing the detection sensitivity for the specific nucleotide sequence. One of the sequence of interest that could serve as a reliable tumor marker is the mRNA encoding telomerase subunits. The telomerase is a ribonucleoprotein functioning as a reverse-transcriptase enzyme in the process of telomeres' synthesis. Its action prevents "end replication problem" that would lead the cell to proliferative senescence or apoptosis. Potentially immortal cells (tumor cells, germ cells) must have the possibility to compensate for the base pair loss. The detection of some essential subunits of this enzyme could serve as a valuable tool in cancer diagnosis. There are at least two essential subunits that are necessary for reconstitution of telomerase activity; RNA component (TR) acting as a template region that binds repeats in telomeres and catalytic subunit (TERT) responsible for the reverse transcription. The catalytic activity of the enzyme is generally regulated through the presence and activity of TERT. The present study was carried out to test the usefulness of hTR and hTERT mRNA detection in the plasma of patients with breast carcinoma.

**Materials and methods:** *Patients:* Twenty-five patients with breast cancer and 7 healthy subjects were included in the study. *Sample collection:* Ten ml of blood in EDTA containing tubes was centrifugated at 880 x g for 10 min and the obtained plasma (4 ml) was centrifugated again at 880 x g for 10 min. Total RNA was extracted from plasma using Trizol LS Reagent. At the first step, the whole RNA was reversely transcribed to a pool of cDNA. At the second step, GAPDH, hTR and hTERT fragments that were amplified from the pool of cDNA by PCR reaction and were amplified once again in the semi-nested PCR using the same primers and conditions as in the first PCR reaction. The PCR products were separated by agarose or polyacrilamide gel electrophoresis.

**Results and discussion:** In the plasma samples collected from healthy volunteers, the control GAPDH was positive in all cases, hTR in 3, whereas and hTERT was negative in all 7 tested cases. Among 25 patients with breast cancer, 23 were hTR and 12 hTERT positive. Two patients who were negative for hTR and hTERT were negative also for the control GAPDH that was otherwise positive in all other tested cases. These results suggest that hTR cannot be used as a tumor marker because it is present in all patients and in more then 40% of samples obtained from healthy volunteers. On the contrary, hTERT may be useful for the detection of circulating specific RNA originating from tumor cells. Since the RNA originating from lymphocytes could lead to false positive results, the quantitative determination with the settled cut-off seems to be more accurate and useful.

# *In vitro* investigations on the variation in induction of apoptosis in gliomas by antidepressants

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Investigations are being carried out in the laboratory to examine the potential efficacy for antidepressants to induce apoptosis in respirationally-compromised glioma cells. This work is associated with the clinical trial of Clomipramine (also presented at this conference). Tumour tissue was collected after patients consented to take part in the clinical trial and was derived from excess tumour tissue collected by surgeons for diagnostic biopsy. Tissue was the cultured to enable in vitro characterisation of tumour. Once a diagnosis of anaplastic astrocytoma (WHO grade III) or glioblastoma multiforme (WHO grade IV) was made patients were entered on to trial.

A test of a cells susceptibility to Clomipramine was made by assessing the effect on oxygen consumption (via an oxygen electrode) and cells viability (via MTT assay). Variability of the effect of Clomipramine on oxygen consumption will be measured and compared to the efficacy in patients and patient subgroups. This model of the apoptitic effect of Clomipramine will be compared to the viability of cell in the presence of similar concentrations of Clomipramine using the MTT assay. Biopsies will be further characterised for multi-drug resistance and GFAP.

Other investigations examining other structurally related tricyclic antidpressants and selective serotoin-uptake inhibitors is progressing in this laboratory. The effect of cathepsin L inhibitors, fetal calf serum and dexamthasone and phenytoin on the induction of apoptosis is being investigated.

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# Electrogenetherapy with *p53wt* combined with cisplatin: an *in vitro* study on human tumour cell lines with different p53 status

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**Introduction:** Electroporation has proven to be a successful method for introducing genes (electrogenetherapy) and chemotherapeutics (electrochemotherapy) *in vitro* and *in vivo* into the cells of various tissues. The tumour-inhibiting p53 protein is a transcription factor and is found in very low numbers in normal cells. Different forms of stress can lead to post-translational modifications in p53 and its stabilisation. The accumulation of p53 triggers a transcription of the genes involved in cell-cycle inhibition or apoptosis. In most types of human cancer p53 remains inactivated due to mutations in the p53 gene or links between p53 and other proteins. Cisplatin is among the most frequently used chemotherapeutics in clinical treatment of cancer. Cytotoxicity of cisplatin and its anti-tumour effectiveness can be greatly increased by electroporation of cells or tumours.

**Aim:** The aim of our study was to evaluate electrogenetherapy with *p53wt* alone or combined with cisplatin on two colorectal (HT-29 and LoVo) and two prostatic (PC-3 and DU145) carcinoma cell lines with different p53 status.

**Materials and methods:** The status of endogene p53 in used cancer cell lines was determined by immunostaining using DO-7 antibody. Cytotoxicity of cisplatin alone, electrogenetherapy and the combination of both was determined by clonogenic assay. Morphological changes and apoptosis were evaluated on cytological preparations, which were stained using the Giemsa's method or with a mixture of acridine orange and ethidium bromide. The degree of apoptosis in the cells was determined by measuring the fluorescence of anexin using flow cytometer.

**Results**: Electrogenetherapy with *p53wt* was dependent on the p53 status of the cell lines used. Electrogenetherapy was the most effective on the PC-3 (*p53 null*) and DU145 (*p53mt*) cells and to the much lesser extent in LoVo cells (*p53wt*). The exception was HT-29 cell line with overexpressed mutated p53, where electrogenetherapy with p53wt was the least effective. Combination of electrogenetherapy and cisplatin resulted in additional cell kill by cisplatin, and was not dependent on the p53 status.

**Conclusion:** Results showed that electrogenetherapy with *p53wt* is feasible and results in comparable cytotoxic effect to viral-mediated p53wt gene delivery. This therapy was effective and dependent on the p53 status of the cell lines used. Combination of electrogenetherapy and cisplatin resulted in additional cell kill by cisplatin and was not dependent on the p53 status in these cell lines.

# Cloning, expression, purification, refolding, and initial biochemical characterization of human tissue kallikreins

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The human tissue kallikrein gene family includes 15 members which are all clustered in a 300 kb region on chromosome 19q13.4. Tissue kallikreins (hK1 to hK15) belong to the serine protease family, most of them displaying trypsin-like activity with exception of hK3, hK7, and hK9 which are assigned to the chymotrypsin-like proteases. Most of the tissue kallikrein genes/proteins have been found to be either under- or overexpressed in certain carcinomas, especially in breast, prostate, testicular, and ovarian cancer. Some members may also be involved in the pathogenesis of Alzheimer's disease. Thus, the tissue kallikreins are interesting biomarkers and may represent novel therapeutic targets for cancer and brain disease.

To biochemically and structurally characterize tissue kallikreins 4 to 15, we isolated their full length cDNA (with exception of hK10) from breast and/or ovarian cancer tissue. Subsequently, the sequence encoding the mature proteins (excluding the signal- and the activation peptides) was amplified, a short DNA sequence encoding the cleavage site for enterokinase (DDDDK↓) added to the 5' end of the various fragments, and the DNA fragments cloned into a bacterial protein expression vector. That way, the fusion genes encode synthetic pro-forms of the various tissue kallikreins with an N-terminally located histidine-tag followed by an enterokinase cleavage site. Transformed *E. coli* cells, induced with IPTG, produced high amounts of recombinant (nonglycosylated) tissue kallikreins, which were purified under denaturing/slightly reducing conditions. Refolding methods were established and active recombinant enzymes produced by enterokinase cleavage. In the case of hK4 and hK13, the generation of the mature N-terminus was verified by protein sequencing. With fluorogenic and/or chromogenic substrates, we demonstrated that all of the initially produced enzymes, hK4, hK6, hK8, hK11, and hK13, display enzymatic activity following activation with enterokinase. Thus, these proteins seem to be suitable for further biophysical and biochemical characterization.

# Passive targeting of cysteine protease inhibitor cystatin into tumour cells by using poly(lactide-co-glycolide) nanoparticles

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Cysteine proteases are a group of enzymes with an important role in intra- and extracellular proteolysis. Several studies reported that these enzymes are capable to degrade extracellular matrix (ECM) and are involved in processes of tumour invasion and metastasis. We have shown recently that both fractions of cysteine proteases, i. e. extracellular and intracellular, are participating in degradation of ECM, therefore, the inhibition of both fractions would have high potential to impair malignant process. Using chicken cystatin as a model inhibitor, a new dosage form, nanoparticles, was prepared and tested for its ability to deliver the drug in the cells by a passive target mechanism.

To envisage this mechanism, cystatin was first labelled with Alexa Fluor 488 fluorescent dye (Molecular Probes, USA), and incorporated in poly(lactide-co-glycolide) nanoparticles by double emulsion solvent diffusion method using technique of low energy input in order to preserve biological activity of the protein drug. NP had size of 300-350 nm diameter, and contained 1.6% of active cystatin, retaining 85% of its starting activity. The internalization of NP was performed on a transformed human breast epithelial cell line, MCF-10A neoT. Incubation of NP with cells was carried out for different incubation period in a humidified atmosphere at 37° C, containing 5% $CO_2$.

Image analysis showed rapid internalization of NP. Initially, green fluorescent spots, representing NP, were located on the periphery of the cells. Later on they were distributed mainly in the perinuclear region, where they aggregated, inducing highly fluorescent image. Some faint diffused staining was also found within the cytoplasm which most likely corresponded to labelled cystatin releasing from NP during the incubation period. On the other hand, a control sample with free cystatin in solution did not yield any fluorescence.

Our results thus indicate that nanoparticles rapidly enter the cells, and that by using NP as a carrier system we can deliver protein drug into tumour cells in much higher yield as compared to passive diffusion of free protein in solution.

# Cystatins and cysteine proteases in murine and human tumors

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Cystatins - extracellular cystatin C (CC) and intracellular stefin A (SA) are natural tight-binding, reversible inhibitors of cysteine proteases - revealed immunomodulatory activity (CC) (1). CC and SA were used as tumor markers in several human tumors (1).

**The aim:** To study CC and SA as possible tumor markers in murine tumors and in human haemoblastosis.

**Methods:** CBA (lymphosarcoma LS), (CBA/C57Bl)J6 (Lewis lung adenocarcinoma) and A/Sn (HA-1 hepatoma) mice were used; treatment included cyclophospamide, CPA, in the doses of 50-150 mg/kg, and carboxymethylated b-1,3-glucan (produced by Chemical Institute, Bratislava, Slovakia), 25 mg/kg, one day before or simultaneously with CPA. CC, SA and cathepsin B concentrations were measured by ELISA kits (KRKA, Slovenia). Cathepsin B activity was determined using fluorigenic substrate Z-L-Arg-L-Arg-MCA with CA-074 as inhibitor (3).

**Results:** Serum CC concentration increased in patients with haemoblastosis (46 - with Hodgkin's and Non-Hodgkin's lymphomas), especially in Hodgkin's lymphoma (102.1 9.42 mol/l) compared to healthy donors (64,3 2,82mol/l) and the levels normalized after antitumor treatment. Murine tumor development was associated with decreased serum CC level (Lewis lung adenocarcinoma, lymphosarcoma LS, HA-1 hepatoma) and increased serum SA concentration (HA-1 hepatoma, Lewis lung adenocarcinoma). In general, changes of CC and SA concentrations in serum of tumor bearing mice were opposite. In tumor tissue of the same groups of mice, very low CC concentration was noted and it increased after effective antitumor treatment by CPA; SA concentration also increased (treatment by CPA of lymphosarcoma LS and by carboxymethylated β-1,3-glucan of HA-1 hepatoma). Both cathepsin B activity and concentration in tumor tissue increased several times during effective antitumor treatment (lymphosarcoma LS, Lewis lung adenocarcinoma). Serum serpin α1-protease inhibitor (α1-PI) activity decreased in all murine tumors studied: in HA-1 hepatoma - up to 70% from the control, in lymphosarcoma LS - 2-times, and in Lewis lung adenocarcinoma - about 2-times. There was no restoration of serum a1-PI activity after effective antitumor treatment.

**Conclusion**: It is concluded, that in models of murine tumors CC concentration in tumor tissue and also in serum can be used as an index of tumor development and the efficacy of therapy, CC changes in tumor bearing mice are opposite to data in human haemoblastosis.

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# Unusual localization and inhibitory properties of cystatin F in U937 cells

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Cystatin F has been recently discovered as an inhibitor of cathepsin L and papain. Its expression is restricted mainly to cells important for immune system. As a difference to other type II cystatins, a large proportion of cystatin F resides inside the cells in lysosomes, where it might have a role in regulating cysteine protease activity in processes of antigen presentation.

We studied *in vitro* inhibition of cathepsins C, F, K, H, V and X with cystatin F using fluorescent synthetic substrates. Endopeptidases, cathepsins L1, F, K, V inhibit cystatin F in the Ki range 0.2 - 0.4 nM, while cathepsin S inhibition was 100-fold weaker and was in the range of cathepsin H. Exopeptidases cathepsins C and X were not inhibited by cystatin F.

To clarify biological significance of inhibition, distribution of cystatin F and localisation with cathepsins mentioned above was visualized using specific polyclonal and monoclonal antibodies in pro-monocyte U-937 cell line. Using confocal fluorescent microscopy, only cathepsins H and X were found to be co-localized with cystatin F. The fact that cystatin F is not localized in the same vesicles as its potential targets suggests a protective role of this selective cysteine protease inhibitor in endosomal/lysosomal vesicles.

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# Cathepsins B, L and D activity and cystatin C concentration in mice with sensitive and resistant to cyclophosphamide variants of lymphosarcoma LS

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Cysteine proteases and their endogenous inhibitors have been shown to participate in processes of tumor growth, vascularisation, invasion and metastasis. Their expression, activity and levels in tumor tissue and extracellular fluids can be used as diagnostic and prognostic markers in cancer patients (1) Serum cystatin C concentration and cathepsins B, L and D activity in tumor tissue in mice variants of lymphosarcoma LS, sensitive and resistant to cyclophosphamide (CPA), have been studied. In comparisson with sensitive variant LS, the resistant variant LS was characterized with more aggressive development: faster tumor growth, poor condition of mice and their shorter survival. Sensitive variant of lymphosarcoma LS had a treatment response to CPA dose 10 mg/kg, while resistant variant responded to 15 times higher CPA dose (150 mg/kg). In the sensitive variant CPA induced apoptosis of tumor cells; In the resistant variant of lymphosarcoma LS apoptosis was less expressed and necrosis of tumor cells predominated. Mice were decapitated 14 days after tumor transplantation (4 days after CPA treatment). Cysteine proteases activity in tumor tissue was measured by fluorimetric method (2) and cathepsin D activity by spectrophotometric method against azocasein. Serum cystatin C concentration was measured by enzyme-linked immunosorbent assay (ELISA, KRKA, Slovenia).

Cathepsins B, L and D activities in tumor tissue of untreated mice with the resistant variant of lymphosarcoma LS weresignificantly lower then their activity in mice with the sensitive variant (2.2-fold lower cathepsin B, 2.5-fold cathepsin L and 2.4-fold lower cathepsin D). Successful treatment resulted in an increase of cathepsins, activities in both variants, the activities in the treated mice with resistant variant remaining lower than in the sensitive variant. Serum cystatin C concentration decreased in mice with both sensitive and resistant variant of lymphosarcoma LS, compared with with the healthy mice, but resistant lymphosarcoma was characterized with lower cystatin C level ($14.9 \pm 1.74$ nmol/l and $10.2 \pm 0.99$ nmol/l, respectively) whereas healthy mice cystatin C level was $23.7 \pm 0.92$ nmol/l. As a result of CPA treatment cystatin C concentration increased to normal value ($21.7 \pm 1.14$ nmol/l) in mice with sensitive variant, whereas in mice with resistant variant there were no changes in cystatin C level after treatment compareed with untreated mice.

We suggest that lower cathepsins activity and cystatin C concentration in resistant variant of lymphosarcoma LS compared with the sensitive variant, may be responsible for the lack of repsonse to usual CPA dose (10 mg/kg). Possibly, low cathepsins activity and serum cystatin C level, are related to the resistance to apoptosis by CPA in this tumor. Therefore, cysteine cathepsins B and L and their endogenous inhibitor cystatin C and aspartic cathepsin D can provide useful information for drug resistance and therapeutic effects in lymophosarcoma LS.

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# Influence of open versus laparoscopically assisted colectomy on plasma VEGF and VEGFR1

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**Introduction.** Minimal invasive colectomy may attenuate surgery related immunosuppression with a potential beneficial effect on postoperative infectious complications and cancer prognosis. This may in part be due to a reduced postoperative inflammation-mediated angiogenic stimulus. The potent proangiogenic factor VEGF and its soluble neutralizing receptor VEGFR1 may be determinants of the process. We studied perioperative plasma concentrations of soluble VEGF (sVEGF) and soluble VEGFR1 (sVEGFR1) in patients undergoing laparoscopically assisted versus open colectomy in order to demonstrate possible differences in trauma related changes in sVEGF and sVEGFR1.

**Methods.** 60 consecutive patients were randomized to surgical technique. Blood samples were drawn preoperatively and 1, 2, 6, 24, and 48 hours after skin incision, and 8 and 30 days after the operation. In addition, blood samples were collected from 150 voluntary blood donors as control. sVEGF and sVEGFR1 were determined in EDTA plasma by ELISA method.

**Results.** Colorectal cancer patients had significantly higher preoperative concentrations of sVEGF and sVEGFR1 than controls ($p<0.003$ for both). There was no difference in sVEGF or sVEGFR1 concentrations through the evaluation period between the two surgical groups ($p=0.15$ and $p=0.83$ respectively), but highly significant intra- and postoperative fluctuations in sVEGF and sVEGFR1 concentrations were found ($p<0.001$ for both).

**Conclusion.** Although significant fluctuation in sVEGF and sVEGFR1 concentrations during the perioperative period was found, we found no difference in the surgical trauma induced fluctuations between the two surgical groups.

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# The role of cathepsin B in invasion and apoptosis of glioblastoma U87cells

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Cathepsin B (CatB) is lysosomal cysteine protease whose expression and trafficking are frequently altered in cancer. Cat B was found in significantly higher levels in mlignant forms of human glioma *in vitro* (1) and *in vivo* (2) and its levels were prognostic for patient's survival (2). However, it is not clear weather this is due to increased invasiveness of malignant tumour cells or to increased resistance to chemotherapy, e.g. to lower apoptotic rate after cytotoxic treatments of the tumours. Therefore, we aim to investigate the contribution of CatB to invasion and apoptosis of brain tumour cells.

Cathepsin B cDNA fragment was excised from plasmid plc343 with XhoI and KpnI restrictases and ligated in antisense orientation in eukaryotic expression vector pcDNA3.1(-). The complete sequence of cathepsin B was excised from plc343 using XhoI and XbaI double restriction and ligated in sense orientation in pcDNA3.1(-). Commercial glioblastoma cells U 87 (AATC) were stably transfected with the vectors using calcium phosphate precipitation method. For further analysis two antisense and one sense clonal cell line with lower and higher intracellular cathepsin B protein concentration, as measured by ELISA, respectively, were selected. The clone transfected with the empty vector, was used as a control. Invasion of the glioma cells *in vitro* was measured by invasion of cells through fibronectin and collagen coated Tran swell filters.

We demonstrated, that the invasion of antisense transfected clones was lower compared to the control and to the maternal cell line U87, whereas the invasion of the sense clones was nonsignificantly higher. The invasion of all brain tumour cells was higher through fibronectin than through collagen and Matrigel. Apoptosis of U87 cells was induced by staurosporine, but we did not get significant differences between the transfectants, using three different methods to determine apoptosis, the differential cellular staining, caspase 3 and 7 determination and mRNA expression of Bcl2 gene. Our study shows that similar to cathepsin L (3), cathepsin B is actively involved in invasion of glioma cells but may not have a role in the intrinsic pathway of apoptosis in these tumour cells, when induced by staurosporine.

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3- Levicar et al. Selective suppression of cathepsin L by antisense cDNA impairs human brain tumor cell .invasion in vitro and promote apoptosis. Cancer Gene Therapy 10, 141-151, 2003-

# Regulation of cathepsin L affects the invasiveness and apoptosis of glioblastoma U87 cells

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Cathepsin L (CatL) is lysosomal cysteine protease implicated in various stages of tumor progression. Elevated expression of CatL may facilitate degradation of extracellular matrix and was found associated with increased malignancy of glioma cells (1). CatL is also suggested to play a role in proteolytic cascades associated with apoptosis. Previously, we have demonstrated, that CatL transfection of primary anaplastic astrocytoma IPTP cells resulted in increased invasion and resistance to apoptosis, induced by staurosporine (2). The main aim of the present study was to investigate the effects of CatL manipulation by the sense/antisense cDNA transfection in brain tumor invasion and apoptosis.

Full length PreProCatL cDNA in the sense and antisense orientation was cloned into mammalian expression vector pcDNA3.1- and the human permanent glioblastoma cell line U87 was stably transfected with this construct, using calcium phosphate precipitation method. Sense and antisense positive clones were separated by specific PCR primers. CatL expression was determined at mRNA, protein and activity level. Two sense clones with the highest CatL expression and two antisense clones with the lowest CatL expression, respectively, were chosen for further analysis. Invasive potential through various substrates (matrigel, fibronectin, laminin and collagen) was determined using the modified Boyden chambers. Staurosporine induced apoptosis was determined with differential staining, caspase activity and mRNA expression of apoptotic genes Bcl2 and Bax.

Although CatL mRNA expression was not significantly altered in the sense and antisense tranfectants, the protein and activity levels of CatL were several folds higher in sense transfected clones and lower in the selected antisense clones, when compared to the (vector transfected) controls. Protein and activity levels of the related cysteine protease, CatB, as well as protein levels of cysteine proteinase inhibitors, stefin A and stefin B were not affected by CatL transfection. Transfection with CatL altered the invasiveness of transfectants Although invasiveness varied with the substrate (higher with fibronectin and matrigel then with laminin and collagen), the selected sense clones were consistently significantly more invasive than the antisense clones. Compared to the control, the ratio between Bax and Bcl2 was elevated in CatL antisense clones, and lower in sense clones. Furthermore, caspase 3 and 7 activity was higher in antisense clones, and lower in sense clones compared to the controls. Our data confirmed that similar to IPTP cells, CatL overexpression is increasing the invasiveness of the glioblastoma cells U87 , and increasing the resistance of the U87 cell to apoptosis, when the intrinsic pathway was triggered by staurosporine.

1- Lah *et al.*, Clinical and experimental studies of cysteine cathepsins and their inhibitors in human brain tumors. Int J Biol. Markers, Vol 15, 1, 90-93,2000

2- Levicar *et al.* Selective suppression of cathepsin L by antisense cDNA impairs human brain tumor cell .invasion in vitro and promote apoptosis. Cancer Gene Therapy 10, 141-151, 2003-

# The role of reduced glutathione in microcystin-LR induced genotoxicity

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Microcystins are the most commonly found hepatotoxic cyclic heptapeptides produced by different freshwater cyanobacterial species such as *Microcystis aeruginosa*. They are potent inhibitors of protein phosphatases 1 and 2A (1), which can result in the disruption of many cellular processes and alteration of the cytosceletal structures. This is directly related to their cytotoxicity and tumor promoting activity (2). Microcystins have been shown to induce DNA damage *in vitro* and *in vivo*, however, the mechanisms of their genotoxic activity remain unclear (3).

In our study we investigated the role of oxidative stress and intracellular reduced glutathione (GSH), which is the principal nonprotein thiol involved in the antioxidant cellular defence in MCLR induced genotoxic effects. Using a fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA) we showed that at nontoxic doses MCLR induced dose and time dependent increase of intracellular reactive oxygen species (ROS) formation in HepG2 cells.

To explore the role of ROS and intracellular GSH in MCLR induced genotoxicity two alternative approaches were used: HepG2 cells were pretreated a) with *N*-acetyl-L-cysteine (NAC; 5mM), a well-established thiol antioxidant and precursor of GSH or b) with D,L-buthionine-[S,R]-sulfoximine (BSO; 150 μM), which depletes non-protein thiols including GSH. The cells were than exposed to different concentrations of MCLR for various periods of time. Pretreatment of HepG2 cells with NAC almost completely prevented formation of MCLR induced strand breaks as shown by comet assay. In contrast, pretreatment of cells with BSO, markedly increased the formation of MCLR induced DNA strand breaks. In cells that were not pretreated with BSO MCLR-induced DNA damage was successfully repaired after 6 hours of treatment while the cells pretreated with BSO were unable to repair DNA damage. These results show that intracellular GSH plays important role in protection against MCLR induced DNA damage. The level of intracellular reduced GSH in HepG2 cells after treatment with MCLR was measured with a fluoroprobe monochlorobimane (mBCl). A significant decrease in GSH content was observed already after 10 min of exposure to the toxin. The level of GSH then started to rise and reached maximum level after 6 hours of exposure. After 8 hours exposure to the toxin, the concentration of GSH reached the control level. We also determined the level of nonprotein sulfhydryls (NPSH). Slight decline of NPSH was observed after 4 hours of treatment which then increased. The highest concentration was determined between 6 and 8 hours of exposure to the toxin. After prolonged incubation of cells with MCLR there were no differences in NPSH concentrations between control and MCLR treated groups.

This results show that ROS formation and GSH depletion constitute early events after treatment of HepG2 cells with MCLR. In this study we also confirmed the evidence, that GSH plays an important role in the detoxification of microcystins and their genotoxic action.

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# Proteomic approach to identification of differentially expressed proteins in two types of stomach cancer

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Two most common types of stomach cancer include intestinal and diffuse type adenocarcinoma. Molecular pathogeneses of both types differ considerably.

Identification of differentially expressed proteins in both types adenocarcinoma of stomach from samples obtained by Laser Capture Microdissection (LCM) is described. Protein extracts were prepared from selected populations of LCM harvested cells from diffuse and intestinal types of gastric tumors and corresponding non-malignant gastric mucosae. Extracts of non-malignant and tumor samples were differentially minimal-labelled, separated and visualized by two-dimensional difference gel electrophoresis (2-D DIGE). Following differential expression analysis, designated differentially expressed protein spots were excised and specifically digested. Protein digests were used for protein identification with mass spectrometry and data base searching. Analysis of differential expression of proteins in tumors vs non-malignant tissues revealed a number of differentially expressed proteins and showed a difference in both tumor types as well. Differentially expressed proteins nclude oncoproteins that may have an important role in molecular pathogenesis of the disease.

The method showed good compatibility and siensitivity between the 2D DIGE system with differential minimal-labelling and mass spectrometry. Proteomic approaches combined with technologies such as laser capture microdissection (LCM) and highly sensitive mass spectrometry methods could be used to identify greater numbers of lower abundance proteins that are differentially expressed between defined cell populations. Such combined approach could lead us to the discovery of new disease biomarkers for the early detection and diagnosis and molecular targets for therapy.

# Cathepsins X, S and cystatin C in sera of patients with B-cell lymphoma

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Malignant lymphomas can be viewed as clonal expansion of anatomically and functionally normal cells of the immune system. They are heterologous group with different epidemiological characterisations, clinical presentations and courses of disease. B-cell lymphomas originate from transformed B lymphocytes and are present commonly as malignant tumours of lymph nodes. They occur in different forms, depending on a stage of lymphoid activation and differentiation. Besides lymph nodes, B-cell lymphomas may affect other organs, such as central nervous system and skin.

In our study we measured the levels of cysteine proteases cathepsins X and S and cysteine protease inhibitor cystatin C in sera of 32 patients with B-cell lymphoma by specific quantitative enzyme-linked immunosorbent assays (ELISAs). In opposite to well known cathepsins B and L, which are involved in degradation of extracellular matrix, invasion and metastasis, cathepsins X and S have been suggested to regulate antigen presentation and immune response. Cathepsins X and S were not increased in sera of patients with B-cell lymphoma in comparison with 30 healthy donors. On the other hand, cystatin C was significantly increased in patients' sera. Moreover, higher levels of cystatin C were associated with patients with relaps of disease. No correlation has been found with other clinical parameters, such as age and stage or with the levels of cathepsins X and S. Our results suggest serum cystatin C as a possible candidate to follow the progression of B-cell lymphomas.

# Quantification of cell hybridoma yields with confocal microscopy and flow cytometry

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The fusion of antigen presenting and cancer cells leads to the formation of hybrid cells, which are considered a potential vaccine for treating cancer. The quality assessment of hybrid cell vaccines is crucial for the introduction of this new treatment. Flow cytometry was the method mostly used in the recent past, since it is faster in comparison to microscopy, which allows the visualization of single cells. Here we describe a rapid confocal microscopy based approach to quantify hybrid cell yields that resulted from electrofusion. Rat phreochromocytoma cell line (PC12) or dendritic cells (DCs) were stained with red and green vital fluorescent dyes and fused by an electric pulse following the establishment of contacts between cells by dielectrophoresis in a high frequency alternating electric field. Examination of single hybrid cells by confocal microscopy exhibited dual red and green fluorescence, and cells appeared yellow. The extent of fusion rate was determined by confocal microscopy in two ways: i) counting dual fluorescent (green and red) cells and ii) by measuring the area of co-localized (green and red) pixels relative to all red or green pixels. Results of both methods showed high degree of correlation ($R = 0.86$). The same samples were analyzed for hybridomas also with flow cytometry. Fusion rates determined with confocal microscopy and flow cytometry showed significant correlation, but with a low correlation coefficient ($R = 0.26$, $P = 0.05$). This is ascribed to the different sensitivities of the techniques used. In conclusion, using confocal microscopy we developed a sensitive and a rapid method to asses the yield of hybridomas in a large number of electrofused cells. This new approach is not specific to the cell types used; it can be adapted for use with other cell types.

# *SYT-SSX* fusion genes and prognosis in synovial sarcoma: a retrospective study of 37 slovenian patients

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**Background:** Synovial sarcoma (SS) is characterized by the t(X;18)(p11.2;q11.2) chromosome translocation, which results in generating either *SYT-SSX1, SYT-SSX2* or, infrequently, *SYT-SSX4* fusion gene. The ratio of *SYT-SSX1 : SYT-SSX2* fusions is close to 2:1 in the majority of studies, and the *SYT-SSX2* fusion has been only rarely observed in biphasic SS. Furthermore, the association of *SYT-SSX* fusion types with prognosis in SS has been confirmed in several studies. Patients with the *SYT-SSX1* fusion had significantly shorter metastasis-free and overall survival than patients carrying the *SYT-SSX2* fusion. In the present study we characterized *SYT-SSX* fusion types and evaluated the impact of two different fusions on clinical course.

**Methods:** Tumor samples of 37 patients with SS were collected from the files of Department of Pathology, Institute of Oncology, Ljubljana. Total RNA was isolated from deparaffinized tissue sections by proteinase K digestion, phenol/guanidine isothiocyanate extraction and isopropanol precipitation. The *SYT-SSX* fusion transcripts were detected by RT-PCR using forward primer for the SYT gene and two reverse primers for *SSX1* and *SSX2* genes, respectively. The primers were chosen to specifically amplify junctional regions of the *SYT-SSX1* and *SYT-SSX2*, thus enabling identification of either type of fusion. The SYT-SSX fusion types were confirmed by sequencing following the standard protocols. Survival rates were analysed using the Kaplan-Meier method. Correlations of the *SYT-SSX* fusion type and other clinicopathological factors to survival were analysed by the log rank test

**Results:** *SYT-SSX1* and *SYT-SSX2* fusion transcripts were detected in 9 (24%) and 25 (68%) of tumors, respectively. Three tumors (8%) were negative for either fusion type. Contrary to some other studies, we could not confirm the association of the fusion type and histology observed previously. The *SYT-SSX1* fusion was confirmed in 2/10 (20%) of biphasic tumors and 7/27 (26%) of monophasic tumors. On the other hand, we found the *SYT-SSX2* fusion in 7/10 (70%) of biphasic and 18/27 (67%) of monophasic tumors. Follow-up data were available for 35/37 tumors. Patients whose tumors had the *SYT-SSX1* showed a trend toward better survival by log rank test, although the difference between survival curves was not significant (P=0,17).

**Conclusions:** In our study, the *SYT-SSX2* fusion was found in 68% and the *SYT-SSX1* fusion in 24% of SS. Thus, the ratio of *SYT-SSX1 : SYT-SSX2* is almost 1:3. To the best of our knowledge, this is the first series of patients with SS with such a large number of *SYT-SSX2* cases regardless of the histologic subtype. Furthermore, we found unusually high proportion of biphasic tumors with the *SYT-SSX2* fusion (70%). Contrary to our expectations and recently published findings we could not confirm the impact of the fusion type on survival of patients with SS.

# Schedule-dependency between vinblastine and adriamycin in EAT tumours in mice

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The design of most combined chemotherapeutic schedules used in patients is based on the data from preclinical studies, and phase I and II clinical studies. Little attention is paid to the timing of drugs or possible interaction of drugs in a particular combined schedule. Both these factors could be crucial for the clinical effect of chemotherapy. The aim of our study was to explore whether antitumour schedule-dependency exists for the combination of Adriamycin (ADR) and Vinblastine (VLB) on intraperitoneal (i.p.) Ehrlich ascites tumours (EAT) in mice. Three days after tumour transplantation, animals were treated with VLB (0.006 mg/kg) or ADR (0.9 mg/kg) alone, VLB followed by ADR, ADR followed by VLB and both drugs given simultaneously. The time interval between i.p. injections of the drugs was 24 h. The cell number was obtained by counting viable cells using Trypan Blue exclusion assay. The fluorescence of ADR was measured by flow cytometry and expressed as a percentage of fluorescent cells and mean intensity of signal. After all various treatment schedules, the DNA distribution pattern was also determined using flow cytometry. In addition, morphology of the tumour cells was evaluated.

At the drug dosage used, ADR was more effective compared to VLB in reducing the number of EAT cells in ascites of CBA mice. None of the treatment combination was more cytotoxic than the treatment with ADR alone. However, the DNA distribution pattern and tumour cell morphology differed from one treatment schedule to another. After ADR, or in the case when ADR preceded VLB, as well as when the drugs were given simultaneously, the cells were enlarged with enlarged nuclei. The DNA measurements of these samples showed an increased G2 compartment, which corresponds to the morphologically observed enlarged nuclei. In contrast, the cells treated with VLB alone, or in the case when VLB preceded ADR, the cells were also enlarged, but multinucleated, which is in accordance with the mechanism of action of VLB on mitotic spindle. DNA measurements of these samples showed cells with very high DNA values with no distinctive peaks. In addition, measurements of ADR accumulation in cells showed no difference between various treatment schedules.

In conclusion, the results of our preliminary study show no schedule-dependency between VLB and ADR, at least not at the time-intervals between the drugs and the tumour model we used.

# Do reactive oxygen species play role in 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) induced genotoxic effects?

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Heterocyclic amines are carcinogens readily formed in protein rich food during heat processing. For heterocyclic amines it is known that following metabolic activation they covalently bind to DNA. Therefore adduct formation has been considered to be a major causal factor of DNA damage by carcinogenic heterocyclic amines. However it has been reported recently, that certain heterocyclic amines (i.e. PhIP and DMBA) are capable to induce oxidative DNA damage in vivo. Oxidative DNA damage formed by endogenous or exogenous ROS is mutagenic and is involved in carcinogenicity.

The aim of the study was to assess whether heterocyclic amine 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) can induce intracellular ROS formation and whether oxidative DNA damage contribute to IQ induced genotoxic effects. Using the DCFH-DA fluoroprobe we showed that in human hepatoma (HepG2) cells IQ induced extensive intracellular ROS formation immediately after the exposure, which increased with time of exposure for up to 6 hours. However short term exposure of HepG2 cells to IQ (up to 6 hours) did not induce DNA strand breaks, which were measured with the comet assay. On the other hand in HepG2 cells exposed to IQ for 24 hours the level of DNA strand breaks was significantly elevated. Digestion of DNA from HepG2 cells treated with IQ for 24 hours with purified formamidopyrimidine-DNA glycosidase (Fpg), which recognizes specific oxidized purines, did not reveal any higher level of DNA strand breaks compared with non-digested DNA indicating that IQ either did not induce oxidation of purines or this lesion has already been repaired by this time.

The observed discrepancy between intracellular ROS formation and lack of DNA strand breaks detection after short term exposure can be due to one of several reasons: 1) the IQ-mediated intracellular ROS did not reach DNA to induce strand breaks or 2) the ROS induced DNA strand breaks were efficiently repaired so that they were not detected with the comet assay. These results suggest that in HepG2 cells the strand breaks detected after 24 hour exposure most likely resulted from covalent binding of IQ metabolites to DNA and that oxidative DNA damage did not play role in IQ induced genotoxic effects.

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